Ultrastructural Characteristics of Endothelial Permeability Pathways in Chronic Hypertension

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SUMMARY This study examined characteristics of paracellular and pinocytotic permeability pathways across the middle cerebral artery endothelium of 12- to 16-month-old spontaneously hypertensive rats (SHR). Interendothelial junctions in SHR, like those of age-matched Wistar-Kyoto controls, were impermeable to lanthanum and horseradish peroxidase (HRP) tracers. Freeze-fracture preparations revealed that interendothelial junctions of chronically hypertensive rats are characterized by a twofold increase over controls in the number of tight junctional strands and the mean apical-basal depth. It is believed that this tight junctional hypertrophy may function to increase adhesive forces between neighboring endothelial cells, and may play a role in the prevention of hypertension-induced paracellular permeability increases. Morphological and tracer studies of pinocytotic pathways indicated that, in the middle cerebral artery, endothelial vesicular transport activity is not increased during chronic hypertension. No evidence was found to indicate the presence of transendothelial permeability channels across control or hypertensive arterial endothelium. Thus, increased transendothelial permeability, commonly observed in acute hypertension, does not appear to occur during chronic hypertension, at least in the middle cerebral artery. Our findings suggest that the arterial endothelium may undergo structural (tight junctional) adaptation in response to prolonged hypertension.

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KEY WORDS • endothelium • tight junctions • hypertension • permeability • horseradish peroxidase tracer • lanthanum tracer • freeze-fracture • spontaneously hypertensive rat

MACROMOLECULES may traverse the vascular endothelium via several selective permeability pathways. In the arterial endothelium, the paracellular pathway (through interendothelial junctions) and the pinocytotic pathway (via vesicular transport activity) appear to be the most important transendothelial routes. Numerous reports have documented widening of paracellular pathways and increased vesicular transport in the arterial endothelium of hypertensive animals. Such transendothelial permeability increases have been implicated as important pathogenic factors contributing to atherosclerosis, hypertensive encephalopathies, and other vascular disease states. Most conclusions concerning hypertension-induced alterations in permeability pathways, to date, have been based on studies of acutely hypertensive animals.

Previous studies have suggested that the arterial endothelium may adapt to prolonged blood pressure increases. These adaptations may represent an important component of the total vascular wall remodeling observed in established hypertension, and may contribute to the protective function subserved by medial changes.

In this paper, we describe the ultrastructural characteristics of paracellular and pinocytotic endothelial permeability pathways after extended periods (over 1 year) of hypertension in rats. We studied the middle cerebral artery of spontaneously hypertensive rats (SHR), a commonly used model of human essential hypertension. Our results indicate that, during chronic hypertension, normal permeability characteristics across the arterial endothelium may be maintained, at least in certain segments. This maintenance of normal transendothelial permeability may be due, at least in part, to structural adaptations of paracellular permeability pathways.
Materials and Methods

Sixty-six male spontaneously hypertensive rats (SHR) and age-matched Wistar-Kyoto control rats (WKY) were used in these experiments. Spontaneously hypertensive rats were obtained from a colony maintained at the University of Iowa and represent inbred descendants of the strain originally developed by Okamoto and Aoki. Both prehypertensive (3-week-old) and chronically hypertensive (12-16-month-old) SHR were used. Blood pressures of chronically hypertensive animals, as determined by the tail plethysmographic method, all exceeded 150 mm Hg (mean = 172.6 ± 9); age-matched WKYs averaged 116.4 ± 6 mm Hg.

All animals were anesthetized with sodium pentobarbital (Nembutal, 50 mg/kg body weight, obtained from Pharmaceutical Services, University of Iowa). Following tracer circulation (where applicable), middle cerebral arteries (MCA) were fixed by immersion or via perfusion through the left ventricle. Initial segments of the MCA (5–10 mm distal to its origin, approximately 10–15 mm in length) were used in all studies.

Thin-Section Protocol

Tissues from four animals (350–400 g) used for routine transmission electron microscopy (TEM) were fixed in 4.0% glutaraldehyde buffered to pH 7.2 in 0.16 M sodium cacodylate buffer (330 mOsm). Following osmication (1.0% in buffer) and en bloc uranyl acetate treatment (3.5% aqueous), tissues were dehydrated and embedded in Araldite (Polysciences, Warrington, Pennsylvania). Thin sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome (Sorvall, Newtown, Connecticut), stained with lead citrate, and photographed on a JEOL 100B TEM (JEOL, Peabody, Massachusetts).

Lanthanum Protocol

For lanthanum tracer studies, tissues from six animals (390–450 g) were fixed via vascular perfusion with a solution composed of equal parts of 2.0% lanthanum nitrate, pH 7.7, and 5.0% glutaraldehyde in 0.32 M cacodylate buffer, pH 7.1, and prepared for TEM as described above. Sections were examined and photographed without poststaining.

Horseradish Peroxidase Protocol

Ten animals (350–440 g) used in horseradish peroxidase (HRP) tracer studies received 20 mg Sigma Type II HRP/100 g body weight (Sigma Chemical Company, St. Louis, Missouri) in 1 ml saline via injection into the inferior vena cava. Following a 5-minute tracer circulation, tissues were fixed in 4.0% glutaraldehyde in cacodylate buffer (60 minutes at room temperature), rinsed five times in 0.05 M Tris-HCl buffer, pH 7.0, incubated in the cytochemical medium (0.05 M Tris-HCl containing 0.15% 3,3'-diaminobenzidine tetrahydrochloride, obtained from Sigma Chemical Company, and 0.02% H₂O₂) in the dark for 90 minutes at 37° C, then processed for TEM as described above. Controls included: 1) incubation of tissues in medium without diaminobenzidine; 2) incubation of tissues in medium without H₂O₂; and 3) incubation of tissues from a non-injected control animal in complete medium. Sections were examined and photographed without poststaining.

Freeze-Fracture Protocol

Tissues from 40 animals 12-16 months of age (335-440 g) and 10 animals 3 weeks of age (33-56 g) were fixed for 10 minutes at room temperature in 4.0% glutaraldehyde in cacodylate buffer, followed by storage for 3-24 hours in 30% glycerol. Freeze-fracture replicas were made with a Balzers BAE 121 or BAF 301 freeze-fracture device. Morphometric estimation of tight junctional morphology (12-16 month group only) was accomplished via application of clear plastic overlays ruled with parallel lines (spacing: 3.2 mm) over electron micrographs (X 60,000) of junctional replicas. Intersections of parallel lines and tight junctional particles were counted and averaged to determine the mean number of junctional strands comprising tight junctions in each group. Distances between intersections of parallel lines and the outermost junctional particles were averaged to determine the mean apical-basal depth of each junction. Since arteries from several rats were often pooled to provide enough tissue for adequate replicas, the value of \( n \) used in statistical analysis was taken as the number of replicas from which the junctions analyzed were photographed. Student's \( t \) test was employed as a test of statistical significance.

Results

Paracellular Pathway Observations

Interendothelial junctions of the middle cerebral artery in both control and hypertensive animals varied in appearance from a simple, abutting type (fig. 1 upper), to a more complex type characterized by varying degrees of cellular overlap (fig. 1 center and lower). Subjectively, SHR junctions tended to exhibit more complex morphologies, although no attempt was made to quantify this difference. Tight junctions appeared in thin sections as areas of close apposition of neighboring membranes (fig. 1), with fusion of outer membrane leaflets. No gap junctions could be positively identified, in MCA endothelium, in the thin-sectioned material.

Treatment of tissues with fixative containing 1.0% lanthanum resulted in the deposition of electron-dense precipitate along the lumenal plasma membrane (fig. 2 upper). Lanthanum filled the luminal plasmalemmal vesicles and, in both age groups of SHR and WKY animals, was confined to the luminal portions of interendothelial clefts. Typically, the tracer was unable to penetrate beyond the first or second luminal tight junctional fusion (fig. 2 upper).

Figure 2. Interendothelial junctions of the middle cerebral artery from chronically hypertensive SHR. Upper photomicrograph: After treatment with colloidal lanthanum tracer. Lanthanum is visible underlying the elongated marginal flap but does not penetrate beyond the first tight junctional fusion (large arrow). No lanthanum deposits were observed within the intercellular space (arrowheads) or in the subendothelium. Unstained. X 57,500. Lower photomicrograph: After 5 minutes of HRP tracer circulation. Reaction product is visible in the vessel lumen, along the luminal plasma membrane, and in the luminal portion of the intercellular cleft. No reaction product is present in the intercellular space (smaller arrows) beneath the first tight junctional fusion (long arrow), or in the subendothelial space. Unstained. X 140,000.
In HRP-treated chronically hypertensive and control rats, distribution of reaction product was identical following the 5-minute tracer circulation. The HRP was readily observed in the vessel lumen and deposited along the luminal plasma membrane (fig. 2 lower), but was never observed in interendothelial (junctional) spaces or in the subendothelium. As with lanthanum, HRP was apparently not able to penetrate endothelial tight junctions beyond the first or second luminal fusion (fig. 2 lower) in either SHR or WKY.

Freeze-fracture replicas of MCA endothelium revealed that these cells are linked to one another by an occluding network of tight junctional particles of approximately 10 nm diameter (fig. 3). In addition, the presence of very small intercalated gap junctions (particle size approximately 8 nm) was observed. Intereendothelial tight junctions from chronically hypertensive animals (SHR 12-16 months old) were characterized by a statistically significant increase in both apical-basal depth and number of strands per junction, as compared to junctions from age-matched WKY controls (table 1). The calculated mean number of strands per unit junctional depth was identical in both groups. Junctional morphology in 3-week-old (prehypertensive) SHR was identical to that of WKY.

**Pinocytotic Pathway Observations**

Pinocytotic vesicles (approximately 70 nm diameter) are a common feature of both control and hypertensive MCA endothelium (fig. 4 upper). Vesicles could be classified as luminal (attached to the luminal plasma membrane), free (unattached), and abluminal (attached to the basal plasma membrane). In both SHR and WKY, abluminal vesicles appeared to outnumber luminal vesicles. Free vesicles were uncom-

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**Figure 3.** Freeze-fracture replica of interendothelial junctions from the middle cerebral artery. Upper photomicrograph: Control animal. In this preparation, the fracture plane has jumped from the exoplasmic face of the basal cell membrane ($E_T =$ tissue $E$ face) to the protoplasmic face of the lateral cell membrane ($P_L =$ lateral $P$ face). $\times 60,000$. Lower photomicrograph: Chronically hypertensive SHR (lateral $P$ face only). The junctions exhibit a greater number of tight junctional strands than do the control junctions above. Asterisks denote intercellular boundary. $\times 60,000$. 

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TABLE 1. Freeze-Fracture Characteristics of the Endothelial Tight Junctions of the Middle Cerebral Artery in 12- to 16-Month-Old SHR and WKY Rats

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Strands per Junction</th>
<th>Apical-Basal Depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>WKY</td>
<td>2.8-4.1</td>
<td>3.52 ± 0.43</td>
</tr>
<tr>
<td>SHR</td>
<td>5.9-7.8</td>
<td>7.52 ± 0.94**</td>
</tr>
</tbody>
</table>

*p < 0.01.
**p < 0.001.

mon in MCA endothelium in both groups. No apparent difference in endothelial vesicle density was observed between normotensive and chronically hypertensive animals.

In freeze-fractured preparations, attached pinocytotic vesicles appeared either as P-face craters or E-face circumvallate papillae, measuring 20–40 nm in diameter (fig. 4 lower). In both SHR and WKY, attached vesicles were predominant in the peripheral, perinuclear, and nuclear cytoplasmic zones, while parajunctional zones appeared devoid of vesicles. Considerable variability was observed in the distribu-

![Figure 4. Upper photomicrograph: Distribution of pinocytotic vesicles across the middle cerebral artery endothelium in WKY rats. Note the relative paucity of luminal and free vesicles. L = luminal vesicles, F = free vesicles, A = abluminal vesicles. X 100,000. Lower photomicrograph: Distribution of attached pinocytotic vesicles in freeze-fracture replica of middle cerebral artery endothelium (tissue E-face). Asterisks denote intercellular boundaries. Attached vesicles are consistently absent from parajunctional areas (PJ). Cell at bottom is devoid of attached vesicles. X 28,800.](http://hyper.ahajournals.org/)
Endothelial permeability in chronic hypertension

In HRP-treated chronically hypertensive and control rats, reaction product was confined to the vascular lumen and to luminal plasmalemmal vesicles (fig. 5). Occasionally, free vesicles located near the luminal plasma membrane were labeled with reaction product. In both SHR and WKY endothelium, abluminal and centrally- or basally-placed free vesicles were typically free of label. No reaction product was visualized in the subendothelial space of either group.

Lanthanum-treated tissues presented a similar pattern of vesicle labeling (fig. 6). Tracer deposits were localized along the luminal plasma membrane and within luminal vesicles. In addition, many free vesicles located near the luminal front of the endothelium also contained lanthanum deposits, indicating that these "free" vesicles are connected, outside the plane of section, to the cell surface. Lanthanum was never observed in abluminal or more basally-located free vesicles (fig. 6 lower), indicating the absence of patent transendothelial permeability channels formed by fusion of vesicles.

**Figure 5.** Portion of SHR middle cerebral artery endothelial cell after 5 minutes of HRP tracer circulation. HRP reaction product is visible within the lumen, along the luminal plasma membrane, and within luminal plasmalemmal vesicles. Free vesicles located toward the luminal aspect of the cell may also be labeled. Abluminal vesicles (A) are free from HRP label, as are basally-located free vesicles. No reaction product was observed in the subendothelium. Unstained. X 84,000.

**Figure 6.** Portion of endothelial cells of WKY rats. Upper photomicrograph: Luminally-placed free vesicles (A and B) and luminal vesicle (C) are seen. X 239,000. Lower photomicrograph: Portion of endothelial cell following treatment with colloidal lanthanum at the time of fixation. Lanthanum is observed within the lumen, and fills some luminally-placed free vesicles (A) but not others (B). Luminal vesicles (C) are consistently labeled. More basally-located free vesicles (D) never contained lanthanum. Unstained. X 239,900.
Discussion

This paper presents an ultrastructural examination of paracellular and pinocytotic permeability pathways across the endothelium of the middle cerebral artery of chronically hypertensive SHR and age-matched WKY control rats. The primary findings of this study are: 1) the MCA endothelium of chronically hypertensive animals possesses intact paracellular pathways, which, like controls, are impermeable to lanthanum and HRP tracers; 2) interendothelial junctions of hypertensive animals demonstrate an approximately twofold increase in apical-basal depth and the number of tight junctional strands as compared to controls; and 3) the pinocytotic pathway across the MCA endothelium of hypertensive animals appears morphologically and functionally identical to controls. It is therefore concluded that the arterial endothelium of chronically hypertensive animals may not be characterized by the increased transendothelial permeability repeatedly documented in models of acute hypertension.** We speculate that the vascular endothelium may undergo structural (tight junctional) adaptation in response to chronic hypertension.

The Paracellular Pathway

The paracellular pathway is considered to be the structural equivalent of the physiological "small pore" of capillaries. This small pore pathway is believed to be the primary route for the passive transendothelial movement of water, ions, and small macromolecules. In peripheral arterial vessels, this pathway is presumed to be operative due to the permeation of molecular tracers into endothelial tight junctions.

The structural variability among endothelial tight junctions in different vascular segments implies inherent differences in normal paracellular permeability. For example, interendothelial junctions in aorta have been shown to be freely permeable to lanthanum (4 nm) and HRP (5 nm) tracer molecules presumably due to the often macular nature of tight junctions in this segment. The cerebrovascular endothelium, on the other hand, possesses extensive ocluding tight junctional networks and consequently is normally impermeable to lanthanum, HRP, or microperoxidase tracers. Due to this structural variability, the present observations on paracellular pathway characteristics of MCA endothelium may not be applicable to aortic endothelium or other arterial segments in which ocluding networks of tight junctions do not exist.

During periods of acute hypertension, focal openings of retinal and cerebral endothelial tight junctions have been reported. Similar studies have documented increased paracellular protein flux across peripheral arterial endothelium. This hypertension-induced increase in paracellular permeability has been attributed to either angiotensin-induced arterial endothelial contraction, a concept now disputed, or to forced overdistention of the vessel wall. In vessels (such as the aorta) with normally "permeable" junctions, an increase in paracellular permeability may also occur as a function of increased transmural driving forces alone, without physical alteration of the existing paracellular pathway.

Recently, Limas et al. studied endothelial permeability to HRP in SHR with early established hypertension (20 weeks of age). Their studies documented an increase in paracellular pathway permeability in the aorta but not in smaller peripheral arteries. Control junctions from similar aortic segments also were permeable to tracer, although to a lesser extent. Increased paracellular permeability in aortic endothelium of SHR, but not in smaller peripheral or cerebral arteries (present study), may reflect structural differences in paracellular pathways, as described above.

Our observations on paracellular permeability through the MCA endothelium of chronically hypertensive animals indicate that, in these vessels, normal permeability (as measured by lanthanum and HRP tracer molecules) is maintained despite elevated blood pressure levels. This maintenance of normal paracellular permeability characteristics during chronic hypertension may be explained, in part, by the protective vascular wall remodeling suggested to occur in animals with established hypertension. This arterial restructuring has been reported to include medial smooth muscle cell hypertrophy and hyperplasia as well as an increased synthesis of medial connective tissue components. We propose that endothelial adaptation, in the form of tight junctional hypertrophy, may be an important structural component of this protective vascular response.

Interendothelial Tight Junctions

The endothelial junctions observed in the middle cerebral artery conform to previous descriptions in comparable vessels in terms of freeze-fracture characteristics and impermeability to lanthanum and HRP tracers. Our use of quantitative freeze-fracture techniques to compare tight junctional morphology is, while very useful, subject to certain considerations. The effects of fixation on tight junctional morphology in freeze-fractured material and the small percentage of junctional membrane available per replica must be considered as possible limiting factors. In addition, the actual relationship between tight junctional strands and points of membrane fusion (as observed in thin-sectioned material) is unclear. Similarly, the relationship between trans-epithelial permeability characteristics and numbers of tight junctional strands is unclear, although it is generally accepted that the number of strands comprising a tight junction does determine, at least in part, permeability through the paracellular route. Other factors, such as the pattern and complexity of the junctional network, the junctional apical-basal depth, the number of strands per unit depth, and,
possibly, unknown features of the junctional particles themselves may also play important roles. In addition to its function as a diffusion barrier, the tight junction also plays an important role in cell-to-cell adhesion. In this regard, the overall numbers of junctional particles and strands observed in replicas may reflect the potential for adhesive forces between neighboring cells.

Our results demonstrate that interendothelial junctions in the MCA of chronically hypertensive animals are composed of a significantly greater number of tight junctional strands as compared to controls. This observation indicates the potential for greater adhesive or supportive forces between neighboring endothelial cells in chronically hypertensive animals. This factor may tend to impair junctional openings induced by either overdistention or endothelial contraction, and may also reduce endothelial desquamation. We speculate that endothelial tight junctional hypertrophy, as observed in this study, may be a primary factor in maintaining normal paracellular impermeability to lanthanum and HRP in the prolonged presence of high blood pressure. The actual physiological consequences of such hypertrophied tight junctions on paracellular permeability to water, ions, or small macromolecules, if any, are complex and beyond the scope of the present investigation. Whether this junctional hypertrophy occurs in the endothelium of all SHR vessels exposed to high blood pressure or in other models of prolonged hypertension remains to be determined.

Tight junctional hypertrophy in endothelial cells subjected to prolonged periods of stress is also interesting from a purely cytological viewpoint. Modulation of the morphology of occluding junctions in in vivo situations has, to our knowledge, been reported only once previously. Other reports have documented tight junctional hypertrophy in response to various chemical mediators. These results suggest that, like other cellular organelles, tight junctions are capable of in vivo reorganization in response to specific cellular requirements.

Pinocytotic Pathway

The endothelial vesicular transport system is presently accepted as the morphological correlate of the "large pore pathway," the predominant permeability route for larger macromolecules. Morphologically similar transport systems in the aorta and other arteries have been previously demonstrated, indicating that the pinocytotic permeability pathway is operative in nonexchange vessels as well.

Following experimental induction of acute hypertension, increased pinocytotic activity has been reported in endothelial cells of the cerebral and peripheral vasculature. The actual cellular mechanisms underlying these pinocytotic pathway alterations are not presently known. Previous studies of the pinocytotic pathway in SHR have yielded contradictory results. Dermietzel and Eichner, in a quantitative freeze-fracture study, found no difference in the number of pinocytotic vesicles in SHR and WKY cerebral capillaries. Hazama et al., on the other hand, have reported an increased vesicular transport activity in SHR cerebral capillaries.

Our results demonstrate the presence of a morphologically identical distribution of pinocytotic vesicles in the MCA endothelium of both control and chronically hypertensive animals. Consistent with previous ultrastructural studies of microvascular endothelium, abluminal vesicles appeared to outnumber luminal vesicles. Free vesicles, indicative of transendothelial transport, were rare in both SHR and WKY. In freeze-fractured preparations of MCA endothelium, attached pinocytotic vesicles were absent from parajunctional zones, thus extending the results of Simionescu et al. to arterial endothelium. Our observation of occasional endothelial cell membranes devoid of attached pinocytotic vesicles confirm similar observations by Schwartz et al. and indicates that the levels of vesicular transport activity may not be uniform throughout a specific population of endothelial cells.

Using HRP tracer techniques, we have demonstrated an absence of transendothelial pinocytotic activity in the MCA of both chronically hypertensive and control animals. Our results revealed the presence of HRP reaction product only in luminal and luminally-placed free vesicles after a 5-minute tracer circulation. This distribution of label is identical to that obtained in lanthanum preparations in which the tracer is applied at the time of fixation. These observations indicate that, despite the presence of morphologically normal vesicular transport systems, appreciable transendothelial movement of vesicles does not occur, at least within this time period; the attached vesicles must be interpreted as being largely static in nature. Our lanthanum observations also revealed that luminally-placed "free" vesicles (such as those labeled with HRP) should not be interpreted as transporting vesicles but rather as abluminal vesicles connected, outside the plane of section, to the surface. Finally, our tracer studies provide no evidence to support the presence of patent transendothelial permeability channels, formed by fusion of pinocytotic vesicles, across the MCA endothelium of either control or hypertensive animals. Such channels have been documented in the microvasculature of the diaphragm and in cerebral capillaries during acute hypertension.

Our demonstration of normal transendothelial pinocytotic permeability in chronically hypertensive SHR contrasts with a number of studies that document increased pinocytosis in the endothelium of acutely hypertensive animals. This discrepancy may be explained in terms of 1) the absence, in chronic hypertension, of those factors responsible for the stimulation of pinocytosis in acute hypertension; or 2) a decreased endothelial response to such factors. Further knowledge of the mechanisms underlying endothelial pinocytosis is essential for our future understanding of these hypertension-related phenomena.
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