TRANSCELLULAR TRANSPORT AS A MECHANISM OF BLOOD-BRAIN BARRIER DISRUPTION DURING STROKE

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1. ABSTRACT

It is well-known that ischemia causes disruption of the blood-brain barrier (BBB), which leads to the formation of vasogenic brain edema. One major mechanism of BBB opening is enhanced pinocytotic vesicle formation that may be induced after transient focal ischemia by several mechanisms, including nitric oxide production, release of neurotransmitters, inflammatory mediators and hemodynamic alterations. In the present study we sought to characterize the extent of pinocytosis in cerebral endothelium during both ischemia/reperfusion (I/R) and elevated intravascular pressure. Transient focal ischemia was induced for 1 hour with 24 hours of reperfusion using the filament occlusion model in male Wistar rats, after which occluded middle cerebral arteries (MCAs) were dissected and mounted on glass cannulas in an arteriograph chamber. This system allowed control over intravascular pressure, measurement of lumen diameter and perfusion with various tracers (Lucifer Yellow and horseradish peroxidase) for measurement of transcellular transport and quantification of pinocytosis using transmission electron microscopy. I/R was found to increase vesicle formation by 166% basolaterally without a change in vesicle formation apically compared to non-ischemic control MCAs at 75mmHg (p<0.01). Similarly, an acute increase in pressure to 200mmHg caused a 78% increase in apical pinocytosis (p<0.05) and a non-significant 42% increase basolaterally. These results were confirmed by permeability measurements using Lucifer Yellow and demonstrate that both I/R and acute elevations in intravascular pressure enhance cerebral endothelial cell pinocytosis. The increase in basolateral pinocytosis during ischemia suggests enhanced efflux mechanisms that may be transporting substances from brain to blood. In addition, since the enhanced pinocytosis after an increase in pressure occurred in isolated arteries in vitro without the influence of metabolic or neuronal factors, these findings demonstrate that elevated intravascular pressure is a primary stimulus for pinocytosis in cerebral endothelial cells.

2. INTRODUCTION

Cerebral edema formation with a subsequent rise in intracranial pressure is the most dangerous complication of stroke, often occurring during the reperfusion period after recanalization of an occluded artery (1). Brain edema is characterized as either cytotoxic or vasogenic depending on whether or not the blood-brain barrier (BBB) is disrupted (2). Increased vascular permeability is considered the most important factor for development of vasogenic edema and is determined by the cerebral endothelial cells that form the BBB (4,5). The morphologic features of cerebral endothelial cells that prevent the extravasation of large and small solutes are the presence of tight junctions that reduce paracellular transport and the low rate of pinocytotic vesicle formation that limits transcellular transport (6,7). While tight junction structure and function has been extensively studied as a mechanism of BBB disruption during I/R (8,9), the contribution of pinocytotic vesicle formation is much less understood, but may be a significant contributor and a potential therapeutic target.

There is considerable evidence that enhanced pinocytosis contributes to cerebral edema formation during pathologic states. Several studies suggest that I/R enhance transcellular transport in cerebral endothelium and contribute to vasogenic edema formation (10-12). In
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In addition, hypoxia has been shown to increase non-specific transport of proteins into the brain via vesicle formation (13). During post-ischemic reperfusion there are several processes that occur that may promote increased vesicle formation, including enhanced nitric oxide (NO) production, inflammatory mediators, and pathologically elevated neurotransmitters (3,14). For example, dynamin-2, a protein associated with the endocytic machinery, has been shown to be directly linked to endothelial NO synthase, the enzyme that produces NO (15). NO has been shown to enhance BBB permeability alone (16-18), and be induced by inflammatory cytokines that can affect the integrity of the BBB (19,20). Lastly, elevated neurotransmitters including norepinephrine have been shown to enhance pinocytosis in cerebral endothelial cells and promote edema formation (21,22).

In addition to inflammatory and neuroexcitatory mechanisms, the effect of I/R on cerebral endothelial cell pinocytosis may also be related to altered cerebral hemodynamics. Acute hypertension during the initial post-ischemic period, leading to increased cerebral blood flow through dilated arteries, is associated with increased vascular permeability (3, 23,24). One of the earliest microscopic findings in an experimental animal model of acute hypertension (i.e., hypertensive encephalopathy) is an increased rate of pinocytosis in the cerebrovascular endothelium, which allows significant passage of fluid and molecules into the brain (25,26). In addition, the reperfusion period following occlusion of a cerebral artery is associated with a period of autoregulatory failure and dilatation that leads to hyperemia (27). Similar to acute hypertension, this hyperemic period is often associated with acute increases in vascular permeability (28,29). A study by Kuroiwa found that opening of the BBB to protein tracers during post-ischemic reperfusion was associated with reactive hyperemia, with the extent of reperfusion correlating with the degree of BBB opening (28). The vasodilation that occurs during post-ischemic reperfusion may also contribute to enhanced transcellular transport, similar to acute hypertension.

In the present study we tested the hypothesis that I/R increases the rate of pinocytosis and transcellular transport in cerebral endothelial cells and investigated the contribution of changes in cerebral hemodynamics to BBB disruption. To test this hypothesis, we used the middle cerebral artery occlusion model to induce I/R on the MCA, after which the MCA was isolated for both electron microscopy and published elsewhere (35). Briefly, the animals were anesthetized via inhalation mask with halothane and oxygen. With the aid of a dissecting microscope, the right carotid bifurcation was exposed and the external carotid artery stump and gently advanced to occlude the origin of the MCA. Successful occlusion of the MCA was confirmed using laser Doppler flowmetry. A skin incision in the right temporoparietal area was made and the microtip of the laser Doppler fiberoptic probe was glued to the skull with Krazy glue. The probe was left in place and cerebral blood flow recorded throughout the surgery. For all experiments, ischemia was induced for 1 hour followed by 24 hours of reperfusion, after which animals were re-anesthetized by sodium Brevital and quickly decapitated. The brain was quickly removed and placed in cold physiologic saline solution (HEPES PSS). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC).

3.2. Pressurized arteriograph system

A branch-free segment of the MCA was dissected and mounted on two glass microcannulas in an arteriograph chamber that allowed intravascular pressure to be adjusted and maintained within the artery via a servo mechanism and pressure transducer connected to the proximal cannula. The arteriograph chamber contained an optical window just below the mounted artery to allow lumen diameter to be measured continually via video microscopy (36). The chamber was filled with HEPES PSS buffer and maintained at 37 degrees Celsius and at a pH of 7.4. This system was used to both perfuse MCAs with either horseradish peroxidase (HRP) for quantification of pinocytosis, or with Lucifer Yellow, to measure vascular permeability, both described in detail below.

3.3. Quantification of pinocytosis by transmission electron microscopy

A total of 10 male Wistar rats weighing between 280 and 300 grams were used for TEM and quantification of pinocytosis. We compared three groups of arteries: 1) MCAs that were non-ischemic and at normal intravascular pressure of 75mmHg (CTL). These vessels are considered to have an intact BBB and therefore a low rate of pinocytosis. We considered this group a negative control. 2) non-ischemic MCAs that were exposed to high intravascular pressure (HP). This group is known to have increased pinocytosis and was considered the positive control. 3) MCAs that were exposed to 1 hour of ischemia and 24 hours of reperfusion at 75mmHg (ISC). This group was considered the experimental group.

After a 20-minute equilibration period at a pressure of 75 mmHg, all arteries developed spontaneous myogenic tone, a property of cerebral blood vessels that allows them to constrict and dilate in response to changes in intravascular pressure (37). The arteries were then perfused with HRP at a concentration of 0.03125 grams per 10 ml of HEPES buffer. The control and ischemic arteries
were fixed with 2.5% glutaraldehyde for TEM after perfusion of HRP at 75 mm Hg. For the HP arteries, intravascular pressure was increased stepwise from 75 to 200 mm Hg while continuously perfusing with HRP. After 1 minute at the high pressure, diameter was recorded and the vessels were chemically fixed for TEM.

Fixation of the arterial segments was accomplished by adding 1 ml of 25% glutaraldehyde to the HEPES bath that contained the mounted arteries. Twenty minutes after the addition of fixative to the bath, arteries were carefully removed from the cannulas and post-fixed in a 1:10 dilution of glutaraldehyde and HEPES for another 20 minutes. Post-fixation, arteries were exposed to 3,3'-diaminobenzidine (DAB, 6mg/mL) for 20 minutes, followed by DAB with 1% hydrogen peroxide for another 20 minutes.

Arteries were subsequently prepared for TEM in a normal manner that included processing the arteries for dehydration with graded ethanol steps, embedding in SPURS resin, and sectioning the vessels longitudinally using an ultramicrotome. All TEM micrographs were taken of the vessel segments randomly at a magnification of 8000X, with a micrograph scale of 23 µm = 1 micron.

TEM micrographs were scanned into a computer and analyzed using SCION Imaging analysis software. Using this program, the length of both the apical and basolateral endothelial cell membrane, the area of the endothelial cells, and the internal elastic lamina thickness (IEL) were measured. The number and location (apical vs. basolateral) of vesicles per length of membrane were analyzed morphologically by two independent observers and averaged. Morphologic assessment of vesicle number and location was accomplished by counting the number of vesicles and noting whether they were located apically or basolaterally, which was clearly distinct. The number of vesicles apically was divided by the length of the apical membrane to obtain a normalized assessment of number of vesicles per length of membrane i.e., number/µm membrane. This procedure was repeated for the basolateral membrane and vesicles located basolaterally.

3.4 In vitro measurement of transcellular transport using Lucifer Yellow

In a separate group of experiments, permeability of the MCA to Lucifer Yellow was determined under conditions of I/R and increased intravascular pressure. MCAs were dissected from either non-ischemic control (CTL, n=6) or after exposure to 1 hour of ischemia with 24 hours of reperfusion (ISC, n=8) using the MCA occlusion model described above. The MCAs were mounted on glass cannulas within the arteriograph chamber and perfused with 0.5mg/mL Lucifer Yellow dissolved in HEPES PSS buffer. Permeability was determined by measuring the clearance of Lucifer Yellow through the vascular wall by sampling the fluid on the outside of the vessel and measuring fluorescence intensity using a fluorescent spectrophotometer, expressed as a change in fluorescence per minute. Once mounted and perfused with Lucifer Yellow, permeability and diameter were measured at different intravascular pressures: 60, 80, 175 and 200 mm Hg. Because all arteries developed spontaneous myogenic tone within the myogenic pressure range at 60 and 80mmHg, but lost tone at the higher pressures due to the increased intravascular pressure that overcomes the myogenic vasoconstriction, we wanted to know if the increase in permeability at the higher pressures was due to loss of tone (i.e., vasodilation) or forced dilatation (i.e., elevated pressure). Therefore, we treated a separate group of arteries with nifedipine (n=6), a dihydropyridine calcium channel blocker that causes vasodilation and loss of tone without a change in intravascular pressure (38).

3.5. Statistical Analysis

Results are presented as a mean±standard error. Differences between groups were determined by one-way analysis of variance (ANOVA) with a post hoc Bonferroni test for multiple comparisons. For quantification of pinocytosis, a total of 10 animals were used with several different endothelial cells in each group imaged for comparison. Therefore, the n-value = number of micrographs per experimental group; each micrograph contained one endothelial cell at a magnification of 8000X. For permeability measurements, only one artery per animal was used and therefore the n-value = animal number. Comparison between groups (e.g., ISC vs. CTL) was determined using ANOVA whereas within groups (e.g., between different pressures) was determined using repeated measures ANOVA.

4. RESULTS

4.1. Enhanced pinocytosis with pressure and I/R

After 20 minutes at 75 mm Hg, CTL arteries developed spontaneous myogenic tone that decreased diameter from 247 ± 12 µm to 179 ± 25 µm. ISC arteries developed somewhat less tone at 75 mmHg compared to control arteries, decreasing diameter from 271 ± 16 µm to 247± 23 µm. Similarly, HP arteries started with a diameter of 283 ± 17 µm that decreased to 256 ± 14 µm at 75 mm Hg during equilibration. Because we increased intravascular pressure to 200 mm Hg in this group to cause forced dilatation (i.e., loss of myogenic tone), diameter increased to 288 ± 18 µm at the higher pressure.

Figures 1, 2 and 3 show electron micrographs from CTL, HP and ISC arteries, respectively. Non-ischemic control arteries showed normal morphology with few pinocytotic vesicles and little transport of HRP into the internal elastic laminae. In contrast, arteries that were fixed at 200 mm Hg showed a tremendous number of pinocytotic vesicles that formed apically, as well as staining of the internal elastic laminae. Arteries that were exposed to ischemia showed the greatest amount of HRP transported into the internal elastic laminae and vesicles both apically and basolaterally.

Morphologic assessment of the number of vesicles per length of membrane showed that elevated intravascular pressure significantly increased vesicle formation in HP vessels, the majority of which were increased apically (Figure 4). Compared to CTL vessels,
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4.2. Vascular permeability to Lucifer Yellow

The diameter of the ISC MCAs did not differ from the CTL arteries at any pressure studied. Figure 5 shows the diameter of CTL and ISC MCAs at pressures of 60, 80, 175 and 200 mm Hg. Notice that the diameter stayed remarkably constant within the myogenic pressure range of 60-80 mm Hg, but that forced dilatation of myogenic tone occurred when pressure was increased to 175 mm Hg. Permeability to Lucifer Yellow was also similar in the ischemic MCAs compared to CTL at all pressures studied (Figure 6). However, pressure had an influence on permeability, increasing the rate of transfer at the higher pressures. CTL arteries increased permeability to Lucifer Yellow 141% when pressure was increased from 60 to 200 mm Hg (p<0.05). Similarly, ISC arteries increased permeability by 83% with the same pressure step (p<0.05).

Because forced dilatation is associated with both loss of tone and increased intravascular pressure, we wanted to know if loss of tone alone at constant pressure would increase permeability without forced dilation. Figure 7 shows the diameter in response to pressure of CTL arteries and arteries treated with the calcium channel blocker nifedipine. Notice that the diameter of MCAs in nifedipine was significantly higher than CTL arteries without this compound, indicating that there was diminished myogenic tone in this group due to calcium channel blockade. The permeability of arteries in nifedipine was also significantly higher than control arteries, suggesting that vasodilation alone increased transcellular endothelial cell transport of Lucifer Yellow (Figure 8).

5. DISCUSSION

The results of the present study demonstrate that both I/R and acute elevations in intravascular pressure significantly increase the rate of pinocytosis and transcellular transport of cerebral endothelial cells. We found that elevated pressure caused a 78% increase in vesicle formation apically and a 42% increase basolaterally, whereas I/R increased pinocytosis only basolaterally by a substantial 166%. These results were confirmed by studies using Lucifer Yellow that showed no difference in permeability between ischemic and non-ischemic vessels, and only an increase at high pressure in both groups. Because permeability to Lucifer Yellow was measured as a clearance through the vessel wall in the apical to basolateral direction, it would be a measure of apical flux rather than basolateral. Therefore, the lack of increase in the ischemic MCA is likely due to the fact that TEM showed no increase in vesicle formation apically, only basolaterally.

The increase in basolateral pinocytosis during I/R suggests that there is an efflux of substances from the brain tissue into the blood. Cerebral endothelial cells are similar to polarized epithelium in that there are distinct transporters and enzymatic functions on apical and basolateral membranes (39). Therefore, the cerebral endothelium serves not only to protect the brain from toxic substances in
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Figure 3. Transmission electron micrograph showing a middle cerebral artery after being exposed to ischemia and reperfusion. There were vesicles located both apically and basolaterally within the endothelial cell (EC), with considerable staining of the internal elastic lamina (IEL). Artery was sectioned longitudinally and imaged at 8000X. Scale bar = 1.0 micron; SMC = smooth muscle cell; ECM = extracellular matrix.

Figure 4. Graph showing location and number of vesicles per length of membrane in endothelial cells of the three experimental groups. Notice there was a greater number of vesicles located apically vs. basolaterally in the control (CTL) and high pressure (HP) groups, with a significant increase apically at the high pressure. Ischemic arteries (ISC) had a significant increase in basolateral vesicle formation without a change apically. (**) p<0.01 apical vs. basolateral for Control and HP; () p<0.05 vs. apical Control and apical ISC (++) p<0.01 vs. Control and HP basolateral.

the blood, but also from substances produced in the brain from entering the bloodstream. For example, the basolateral membrane contains neurotransmitter degrading enzymes that can inactivate neurotransmitters released during neuronal activity (39,40). It is possible that high levels of neurotransmitter produced during ischemia may contribute to the substantial increase in basolateral pinocytosis noted in this study since several neurotransmitters are known to promote pinocytosis directly (21,22). Other mechanisms are possible, however. In addition to neurotransmitter degrading enzymes, p-glycoprotein, a highly conserved membrane protein, functions as an active efflux pump to remove drugs and toxins from cells, has been found on the basolateral membrane of cerebral endothelial cells and appears to transport substances across the endothelium in the basolateral to apical direction (41,42). Other proteins are also known to be transported from the brain into the bloodstream, including S100 that is used as a peripheral marker of BBB disruption during stroke (43).

While previous studies in situ have shown enhanced pinocytosis after induction of acute hypertension by infusions of norepinephrine or angiotensin II (26,44), this is the first study to specifically demonstrate that increased intravascular pressure alone is a primary inducer of pinocytosis. Because vessels were studied isolated and cannulated, the influence of metabolic and neuronal factors were not present. Intravascular pressure was increased without inducing hypertension by pharmacologic means (e.g., norepinephrine, angiotensin II), thereby eliminating the effect of any pharmacologic agents on vesicle formation. Several neurotransmitters and hormones have been shown to directly increase the rate of pinocytosis in cerebral endothelium (21,22) and therefore it is not known whether it is the acute hypertension or the presence of pinocytosis-inducing agents that caused the increase. The results of the present study demonstrate that intravascular pressure alone is an important contributor to pinocytotic vesicle formation and suggest that vesicle formation in cerebral endothelial cells is sensitive to physical stimulation (i.e., pressure).

Although the mechanism for this mechanotransduction process has not been established, it is possible that dynamin, a protein involved in pinocytotic vesicle formation, has a key role. Dynamin is a GTPase attached to cortical actin and is a key component in the endocytotic machinery (45,46). This protein, located at the neck region of vesicles, is mechanosensitive (46,47). Its activation cleaves vesicles from the apical membrane, providing a potential mechanism for regulation of vesicle formation by mechanical stimulation (i.e., pressure). While the results of this study cannot determine the exact mechanism of pressure-induced pinocytosis, a mechanosensitive enzyme that is involved in regulation of vesicle formation is a likely candidate and deserves further investigation.

The results of this study also demonstrated that vasodilation alone can increase transcellular transport, as demonstrated by increased permeability to Lucifer Yellow in arteries that were treated with nifedipine to inhibit smooth muscle contraction. Nifedipine is a L-type calcium channel blocker and smooth muscle relaxant and was used to inhibit myogenic tone (38). Because endothelial cells do not contain L-type calcium channels, a direct effect of nifedipine on endothelial cell permeability is unlikely. Therefore, vasodilation appears to increase transcellular transport in cerebral endothelial cells. While previous studies have suggested that vasodilation increased
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**Figure 5.** Graph showing the diameter vs. pressure of control and ischemic middle cerebral arteries. Notice that at pressures within the myogenic pressure range (60 and 80 mm Hg) diameter remained constant, however, at elevated pressures forced dilatation of myogenic tone occurred causing significant vasodilation.

**Figure 6.** Graph showing the permeability to Lucifer Yellow of control and ischemic middle cerebral arteries. Notice that permeability remained constant at pressures within the myogenic pressure range, but increased at the higher pressures with forced dilatation. * p<0.05 vs. at 60 and 80 mm Hg.

cerebrovascular permeability (48-50), the mode of solute and fluid transport could not be defined in those studies. In the present study, we specifically investigated the effect of vasodilation on transcellular transport using Lucifer Yellow. This compound is too polar to diffuse through the cell and has been used extensively to investigate transcytosis (30,51). While these findings do not rule out a role of paracellular transport as a mechanism of enhanced permeability with vasodilation, our results clearly demonstrate that vasodilation alone increases transcellular transport in cerebral endothelial cells.

The importance of this finding is that vasodilation during post-ischemic reperfusion is a common occurrence that leads to hyperemia. Hyperemia during the reperfusion period after recanalization of an occluded artery has been associated with BBB disruption and significant edema formation (27-29). In addition, substances produced during ischemia may also influence permeability via their vasodilatory mechanism. For example, large amounts of NO are produced within minutes of MCA occlusion (52). NO is an ubiquitous intercellular signaling molecule synthesized in several cell types including neurons, endothelium, and macrophages from the amino acid L-arginine by the enzyme nitric oxide synthase (53). NO is involved in a wide variety of physiologic functions such as platelet inhibition, inflammation, and neurotransmission (53-56). Being a potent vasodilator, NO contributes to normal vascular tone and blood flow regulation (57). However, NO is also cytotoxic. Macrophages kill bacteria and tumor cells by releasing high amounts of NO and increasing evidence suggests that NO
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Figure 7. Graph showing the diameter vs. pressure of control middle cerebral arteries and arteries treated with nifedipine. Notice that arteries in nifedipine had larger diameters and did not respond to pressure with myogenic activity, indicating diminished myogenic tone compared to control arteries. ** p<0.01 vs. control; *p<0.05 vs. control.

Figure 8. Graph showing the permeability to Lucifer Yellow of control middle cerebral arteries and arteries treated with nifedipine. Notice that arteries in nifedipine had enhanced permeability at all pressures compared to control arteries. * p<0.05 vs. control.

released during cerebral ischemia contributes to brain injury during reperfusion (58,59). The results from this study suggest that another mechanism of damage by NO production during ischemia may be vasodilation during reperfusion.

6. SUMMARY

In conclusion, this study demonstrates that I/R and acute increases in intravascular pressure enhance pinocytotic vesicle formation in cerebral endothelial cells. The increase in pinocytosis in response to pressure was greater apically, suggesting a flux of fluid and proteins from the blood into the brain. Since enhanced pinocytosis was demonstrated in isolated arteries after an acute increase in intravascular pressure, these results suggest that pressure alone is a primary stimulus for vesicle formation. The increase in vesicle formation during I/R was found to be limited to the basolateral membrane, suggesting that efflux mechanisms are activated during I/R that may be a protective mechanism to clear toxic substances from the brain.

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