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Prevention of Oxidative Injury Associated with Thrombolysis for Ischemic Stroke

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Abstract

Although treatment of ischemic stroke focuses on re-establishing blood flow to the brain (e.g., thrombolysis), delayed reperfusion may be associated with oxidative damage to brain capillary endothelial cells, resulting in cerebral bleeding and death (hemorrhagic transformation). The goal of this study was to define cellular mechanisms responsible for reperfusion injury to brain capillaries, and to provide a rationale for more effective treatment of stroke. Mechanisms of oxidative injury to cerebral capillary endothelial cells were measured in the presence and absence of experimental inhibitors to define the roles of transport and metabolic pathways. *In vitro* experiments provided evidence that: (1) intracellular calcium is elevated in brain capillary endothelial cells following simulated transient ischemia and reperfusion, due to reverse movement of Na/Ca exchange; (2) a simultaneous increase of calcium and reactive oxygen species (ROS) during re-oxygenation causes mitochondrial dysfunction, thus initiating apoptosis and loss of brain capillary integrity. *In vivo* studies showed that γ -glutamylcysteine (an antioxidant precursor of glutathione) and the experimental compound KB-R7943 (inhibits reverse movement of Na/Ca exchange) protect brain capillary endothelial cells when co-administered just before reperfusion following transient ischemia. The data indicate that these agents may be useful in preventing oxidative injury associated with thrombolysis for ischemic stroke.

Keywords: glutathione, gamma-glutamylcysteine, antioxidant, stroke, thrombolysis, hemorrhagic transformation

1. Introduction

Cerebrovascular stroke is a leading cause of death and disability in the world [1, 2]. Strokes may be ischemic or hemorrhagic, but most (ca. 85%) are due to interrupted blood flow to the brain, resulting in hypoxia [2]. Thus, the treatment for cerebral ischemia accompanying stroke includes therapies to re-establish blood flow using a thrombolytic agent [3–8]. Delayed reperfusion following cerebral ischemia (>3–4.5 hours) may cause damage to brain capillary endothelial cells [9–11] that can lead to cerebral bleeding and death [12, 13], a process called *reperfusion injury* leading to *hemorrhagic transformation* [14, 15]. Recent evidence has verified that using tissue plasminogen activator (tPA) to dissolve clots is an effective treatment for ischemic stroke, if administered prior to the 3–4.5 hour interval [11, 16, 17]. However, only about 5% of patients with cerebral ischemia arrive at the hospital in time to be treated without causing vascular damage and cerebral hemorrhage, and

a significant number of eligible patients bleed anyway [18]. Thus, preventing injury to the cerebral vasculature when using a thrombolytic agent to induce reperfusion is of great importance. In this study, we provide evidence that reperfusion injury is associated with *oxidative* damage to brain capillary endothelial cells in the presence of elevated calcium. Furthermore, we show that the antioxidant γ -glutamylcysteine [19, 20] (a precursor of glutathione) together with an agent to prevent calcium sequestration inhibit oxidative injury to brain capillaries when co-administered immediately prior to inducing reperfusion for ischemic stroke.

2. Materials and methods

To understand the mechanisms responsible for reperfusion injury to cerebral blood vessels, pertinent transport properties were first defined in isolated plasma membranes derived from brain capillary endothelial cells (i.e., the blood-brain barrier). This was followed by examining the effects of simulated ischemia and reperfusion on cultured blood-brain barrier endothelial cells, and testing the utility of experimental drugs in preventing cellular damage. Finally, the experimental therapeutic approach was tested *in vivo*, using the middle cerebral artery occlusion technique in rats to simulate ischemic stroke.

2.1 *In vitro* studies

2.1.1 *Preparation of brain capillary endothelial membrane vesicles*

Brain capillary endothelial cells are polarized and possess tight junctions (i.e., zonula occludens) [21]. Luminal (blood-facing) and abluminal (brain-facing) membrane vesicles were isolated from bovine brain capillary endothelial cells by methods that we have described in detail [10–12, 22, 23]. Briefly, cerebral capillaries were isolated from cow brains by homogenization, differential centrifugation, and separation on a column of glass beads. Following mild treatment with collagenase, the capillaries were further homogenized, and the endothelial membranes were separated in a discontinuous Ficoll gradient.

2.1.2 *Transport measurements in membrane vesicles*

The methods for quantifying transport measurements using membrane vesicles from brain capillary endothelial cells have been published by us [8, 9, 24–26]. Rates of substrate uptake by luminal and abluminal membrane vesicles were determined using radiolabeled tracers and a rapid filtration technique [27, 28].

2.1.3 *Western blot analysis of membrane vesicles*

NHE1 (Na/H antiporter, isoform 1) and NCX1 (Na/Ca exchanger, isoform 1) were identified in isolated plasma membranes from bovine brain capillary endothelial cells using immunoblotting with mouse monoclonal antibodies (Chemicon) and horseradish peroxidase-conjugated goat anti-mouse antibody, as previously described [29, 30]. The bands were analyzed by laser scanning densitometry.

2.1.4 *Polymerase chain reaction analysis of cerebral capillaries*

After mRNA was isolated from bovine cerebral capillaries [31], first-strand cDNA was synthesized using oligo-dT and AMV reverse transcriptase (Promega or

Invitrogen), and sense and antisense primers (Sigma) were used to generate PCR products for NHE1, Na/K ATPase $\alpha 2$, and Na/K ATPase $\alpha 3$, as previously described [31, 32]. Sequencing was performed on both strands, using a commercial service. Quantitative Western blotting was done as previously described [30]. The bands were scanned and quantified using NIH Image software.

2.1.5 Culturing cerebral capillary endothelial cells

Cultured cerebral capillary endothelial cells were used as an *in vitro* model to measure the effects of simulated ischemia and reperfusion on blood-brain barrier function [33–37]. The capillary endothelial cells were isolated from bovine brain using the method of Meresse *et al.* [33], or purchased from Cell Systems Corporation (Kirkland, Washington). Cells were grown, maintained, seeded, and incubated in the presence of an astrocyte conditioned medium supplemented with cAMP, as previously described [33–37].

2.1.6 Simulating ischemia and reperfusion using cultured cells

Cultured brain capillary endothelial cells were exposed to conditions simulating ischemia and reperfusion by incubating first at 37°C in an ischemic medium (without glucose, pH 6.8) equilibrated with an atmosphere of 95% N₂ and 5% CO₂, followed by simulated reperfusion in a control medium (5.6 mM glucose, pH 7.4) equilibrated with room air and 5% CO₂ [38, 39]. To provide a constant environment, the cells were maintained in sealed chambers (Billups-Rothenberg, CA) pre-equilibrated to the desired atmospheric conditions [39].

2.1.7 Measuring intracellular calcium and sodium concentrations

Intracellular calcium was quantified in cultured cerebral capillary endothelial cells by using a fluorescent probe and confocal laser microscopy, as previously described by us [39]. Cells were preloaded with 5 μ M Fluo-4 [39] and treated under conditions of ischemia and reperfusion as described above. Calcium concentration was quantified by measuring emitted fluorescence [39, 40] at a wavelength of 494 nm in 50 randomly chosen (computer-assisted) cells, representing each treatment.

Intracellular sodium in cultured cerebral capillary endothelial cells was measured as previously described by us [39]. Cells were pre-treated with Sodium Green (5 μ M), and the fluorescent signal was quantified by fluorescence microscopy. Measurements were made from 50 randomly chosen cells representing each treatment.

2.1.8 Measuring actin stress fibers in cultured cells

Cerebral capillary endothelial cells were grown on coverslips and exposed to conditions simulating normoxia, ischemia, and reperfusion as described above. Following treatment, the monolayers were washed in phosphate buffered saline (pH 7.4), fixed for 5 minutes in 3.7% buffered formaldehyde at room temperature, and rinsed again with the buffer [41]. A mixture of phalloidin (0.05 mg/ml buffer) and 1% dimethyl sulfoxide was added to the cells for 40 minutes at room temperature, in a humidified chamber. Following staining, the coverslips were washed with buffer and mounted in a mixture of 30% glycerol in 70% buffer (vol/vol). To determine the effects of calcium-mediated cytoskeletal activation, the cells were incubated in the presence of a myosin light chain kinase inhibitor (0.1 μ M, Sigma).

The tissue was observed and evaluated using laser confocal microscopy, at a thickness of 4 microns.

2.1.9 Measuring permeability of cultured cells

To quantify functional injury to brain capillaries, ^{14}C -sucrose permeability was measured across a monolayer of cerebral capillary endothelial cells [42], under conditions simulating ischemia and reperfusion as described above. Cyclosporin A (1 μM) was used as an inhibitor of the mitochondrial permeability transition [43, 44], to determine if this process is associated with reperfusion injury to the endothelium.

2.1.10 Measuring caspase activity of cultured cells

Caspase 3 activity was measured fluorometrically using an oncogene caspase-3 activity kit, as previously described [39]. Cultured blood-brain barrier cells were lysed, and the lysate was added to the caspase substrate: L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide (DEVD) tagged with the fluorescent molecule 7-amino-4-trifluoromethyl coumarin (AFC). Cleaved AFC was fluorescent, and was quantified at an excitation wavelength of 390 nm, and an emission wavelength of 510 nm.

2.1.11 Measuring lysis/death of cultured cells

Lactate dehydrogenase (LDH) release was used to determine cell lysis, and was measured colorimetrically at a wave-length of 490 nm with a commercial kit (Promega). The effectiveness of various antioxidants [19, 20, 45] in preventing cell death was tested by incubating the cells in the presence (1 mM) and absence of glutathione (GSH), N-acetylcysteine (NAC), and gamma-glutamylcysteine ($\gamma\text{Glu-Cys}$).

2.2 In vivo studies

2.2.1 Middle cerebral artery occlusion

Ischemia and reperfusion were simulated in rats using the middle cerebral artery occlusion (MCAO) technique [46], by placing a thread in the left cerebral artery to reduce blood flow in the left hemisphere, and withdrawing it to re-establish circulation. Briefly, adult female Long-Evans rats (250–300 g) were anesthetized with isoflurane (5% induction and 2% for maintenance). The pterygopalatine artery and branches of the carotid artery were cauterized on the left side, after which a 3 centimeter length of 3.0 Dermalon suture (blunt tip) was introduced in a retrograde direction into the external carotid artery. It was advanced cranially in the internal carotid artery for 23 mm, as measured from the bifurcation of the common carotid artery. This model reduced cerebral blood flow to about 10% of control in the core of the ischemic area. Reperfusion was achieved by withdrawing the thread into the external carotid artery.

2.2.2 Measuring brain capillary mitochondrial morphology

Mitochondrial damage indicative of reperfusion injury was measured in cerebral capillary endothelial cells of rats exposed for 1 hour to cerebral ischemia, followed by 24 hours of reperfusion. Cerebral ischemia and reperfusion was accomplished

using the MCAO model of ischemic stroke. Stroked animals not treated with drugs were infused intravascularly (IV, femoral vein) with 1 ml of physiological saline approximately 1 minute prior to initiating reperfusion. Treated animals were co-administered γ Glu-Cys (400 mg/kg) and KB-R7943 (10 mg/kg) IV in 1 ml of physiological saline 1 minute before reperfusion. The mitochondrial permeability transition was assessed by measuring a typical change in ultrastructural morphology characterized by swelling [47]. Following 60 minutes of transient ischemia and 24 hours of reperfusion, the animals were killed, and brain cortical tissue adjacent to the putamen was sampled and treated with a fixative and prepared for electron microscopy. Fixation involved 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer, and tissue blocks were osmicated, dehydrated in an ethanol series, cleared in propylene oxide, and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined on a Jeol JEM-1230 (HC) electron microscope. Measurements of mitochondrial cross sectional area in cerebral capillary endothelial cells were determined by examining electron micrographs and using computer-assisted morphometry. Briefly, random samples including blood-vessels from the outer cerebral cortical zones of the ipsilateral and contralateral sides were photographed for each animal. Five random samples included 3 blood vessels, 9 endothelial cells, and 45 mitochondria from each of 4 animals per treatment. These samples were measured by outlining the mitochondria with an electronic pen, and compiling the data with commercial stereological software (Neurolucida).

2.2.3 Measuring brain apoptosis

Evidence of apoptosis in the cerebral cortex of the above animals exposed to ischemia/reperfusion (1 hour/24 hours) was determined by using the Apop-Tag Kit (Oncor). Tissue sections were randomly sampled from the cortex, and prepared for immunocytochemistry using the Tunnel assay [48]. The number of stained nuclei were quantified per unit area, as determined with the Neurolucida program.

2.2.4 Measuring neurological behavior

After 1 hour of ischemia and 24 hours of reperfusion, the above animals were observed for obvious neurological deficits indicated by behavioral changes. Attention was focused on motor deficits, torticollis, and obvious paresis.

2.3 Analysis of the data

For the measurements collectively described in this study, values were expressed as an average \pm SD or SE. Means were compared by Student's t test, a paired t test, or an ANOVA, depending upon the conditions. Mean values were considered significantly different if the probability of their being the same was 0.05 or less.

2.4 Vertebrate animals

Plasma membrane vesicles derived from cerebral capillary endothelial cells were isolated from cow brains obtained at a local slaughterhouse immediately after death. The cows were killed for food according to Federal and State laws, and not for the purposes of these experiments. Cultured cerebral capillary endothelial cells originated either from the fresh cow brains described above, or were purchased commercially (Cell Systems Corporation). The middle cerebral artery occlusion procedure *in vivo* was done using rats in the Blood-Brain Barrier Lab at Oregon

Health and Science University (OHSU), Portland, OR. OHSU is in full compliance with Federal and State statutes regarding the use and care of vertebrate animals in research. The animal care facility is AAALAC accredited, and all rat maintenance, treatment, recovery, and euthanasia procedures were approved for the reported middle cerebral artery occlusion studies.

To achieve middle cerebral artery occlusion, female Long Evans rats (250–300 grams) were anesthetized with isoflurane inhalant (5% induction, 2% maintenance). Analgesia was provided by administering butorphanol (0.05–2.0 mg/kg SQ) as needed, and seizures were controlled with diazepam (5 mg/kg) if necessary. Animals were monitored daily by staff veterinarians. Upon completion of the experiments, the rats were euthanized after inhalant anesthetic induction with an intracardiac overdose of sodium pentobarbital, or if symptoms necessitated earlier sacrifice. This method of euthanasia is consistent with requirements of the Panel of Euthanasia of the American Veterinary Medical Association.

3. Results

3.1 Evidence for the presence of NHE1, NCX1, and Na/K ATPase

3.1.1 Sodium flux studies

^{22}Na uptake was measured in membrane vesicles from brain capillary endothelial cells, and revealed both saturable and unsaturable components (**Figure 1**). In the presence of specific inhibitors, three transport pathways were found, including: (a) a low affinity ($K_m = 52 \text{ mM}$), high capacity, dimethyl amiloride (DMA, $100 \text{ }\mu\text{M}$) sensitive sodium carrier, indicative of Na/H antiport; (b) a high affinity ($K_m = 4.6 \text{ mM}$), low capacity, DMA resistant carrier; and (c) a non-specific phenamil inhibitable cationic channel ($K_d = 1.7 \text{ }\mu\text{l/mg/min}$).

3.1.2 Immunoblotting

Immunoblotting using specific antibodies (Chemicon) to NHE1 (Na/H antiporter, isoform 1) and NCX1 (Na/Ca exchanger, isoform 1) revealed that both proteins are present in isolated luminal and abluminal membranes derived from

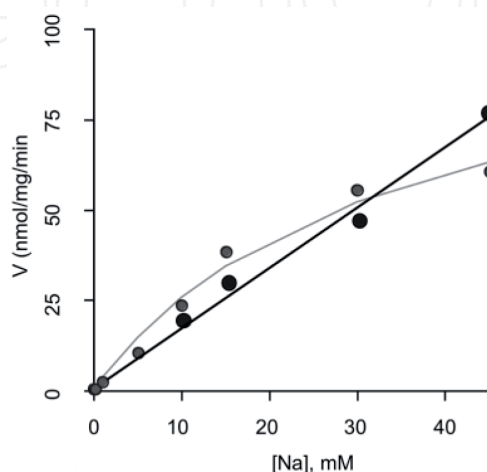


Figure 1.

Both saturable and unsaturable ^{22}Na uptake was measured in plasma membrane vesicles derived from bovine brain capillary endothelial cells. A saturable component ($K_m = 52 \text{ mM}$) was dimethyl amiloride (DMA) sensitive ($100 \text{ }\mu\text{M}$), indicative of a sodium-hydrogen ion exchanger.

brain capillary endothelial cells. NHE1 appeared as a single band with a molecular weight of 106 kD. NCX1 displayed the usual two bands at 43 kD and 150 kD.

3.1.3 PCR studies

Messenger RNA was prepared from isolated bovine brain capillaries, and RT-PCR was performed with specific primers identifying NHE1, Na/K ATPase α_2 , and Na/K ATPase α_3 . The expected PCR products were found for NHE1 (**Figure 2**) and Na/K ATPase α_3 , and sequencing demonstrated a high level of homology to the human transporters (>90%). Na/K ATPase activity was previously measured by us in luminal and abluminal membrane vesicles derived from endothelial cells forming the blood-brain barrier [25]. Activity was found to be present predominantly at the abluminal membrane domain, and was characterized by a ouabain binding constant ($K_d = 25 \pm 3$ nM) typical of either the α_2 or α_3 isoform [49]. The PCR studies reported in the current study confirmed that it is the α_3 isoform.

3.2 Evidence that Na/H exchange elevates intracellular sodium

Intracellular sodium concentration was measured in cultured bovine brain capillary endothelial cells exposed to conditions simulating ischemia and reperfusion. **Figure 3A** shows that intracellular sodium was elevated ($P < 0.05$) during ischemia (120 minutes), and remained elevated during ischemia followed by reperfusion (90/30 minutes). Inhibiting the Na/H antiporter with 100 μ M dimethyl amiloride (DMA) completely prevented the elevation of intracellular sodium observed during ischemia/reperfusion (90/30 minutes), indicating that its activity was responsible for the sustained increase in intracellular sodium observed during ischemia and reperfusion.

3.3 Evidence that Na/Ca exchange elevates intracellular calcium

Intracellular calcium was measured in cultured bovine blood-brain barrier cells exposed to conditions simulating ischemia and reperfusion. **Figure 3B** shows that calcium was significantly ($P < 0.05$) elevated during ischemia (120 minutes), and remained elevated during ischemia followed by reperfusion (90/30 minutes). Inhibition of the reverse mode of Na/Ca exchange (20 μ M KB-R7943) reduced ($P < 0.05$) the level of intracellular calcium observed following reperfusion. Calcium uptake was also significantly inhibited with DMA (100 μ M, not shown), indicating that both activation of Na/H antiport and reverse movement of Na/Ca exchange contributed to the sustained elevation of calcium.

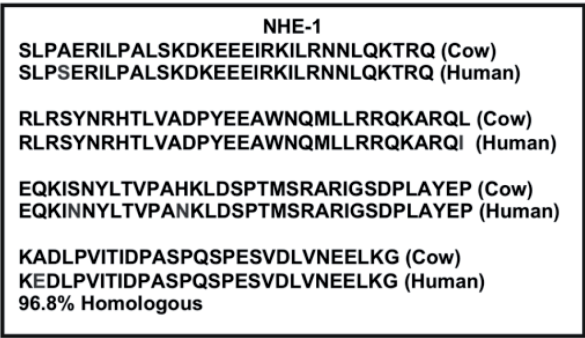


Figure 2.
The RT-PCR product for NHE1 was derived from bovine brain capillary endothelial cells, with a high homology (96.8%) for the respective isoform in human tissue.

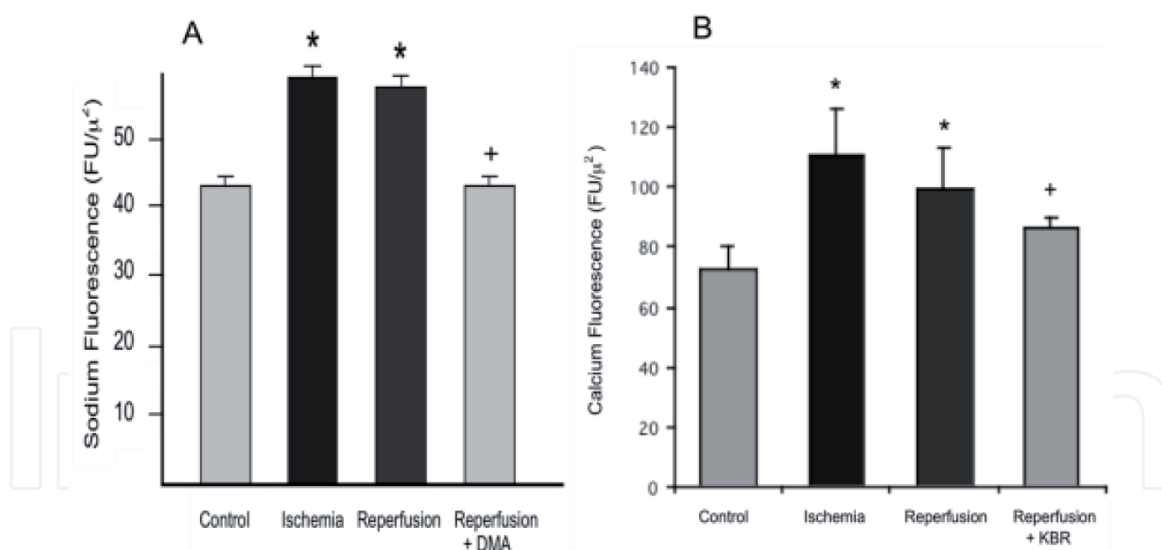


Figure 3.

*Simulating ischemia-reperfusion in cultured brain capillary endothelial cells resulted in a significant increase in intracellular sodium (panel a) during ischemia (120 minutes), that was maintained during ischemia followed by reperfusion (90/30 minutes). The rise observed during ischemia/reperfusion was prevented by inhibiting Na/H exchange (100 μM DMA). The same results were observed when measuring intracellular calcium (panel B). The rise in calcium during ischemia/reperfusion was significantly reduced with a specific inhibitor of the reverse movement of Na/Ca exchange (20 μM KB-R 7943). Intracellular calcium was also significantly reduced by DMA (100 μM (not shown)). *P < 0.05, different from control; + P < 0.05, different from ischemia/reperfusion. Values are mean ± SD. Measurements are made from 50 cells randomly chosen to represent each treatment.*

3.4 Evidence for calcium-mediated alteration of the cytoskeleton

Cultured blood-brain barrier endothelial cells were exposed to conditions simulating ischemia and reperfusion, as described above. Following treatment, the cytoskeletal component actin was stained with phalloidin (0.05 mg/ml) [41] and examined using confocal laser microscopy. Incubating cells for 120 minutes under control conditions revealed the usual configuration of actin, which forms an organized ring of fibrils just inside the plasma membrane. Incubating under ischemic conditions either did not alter this configuration at all, or had a relatively minor effect on cytoskeletal arrangement. However, exposing the cells to ischemic conditions for 90 minutes, followed by 30 minutes of control treatment (reperfusion) caused a remarkable reorganization of the actin (**Figure 4**), which assumed the usual stress fiber configuration associated with damage and increased permeability characteristics [44]. An inhibitor of calcium activated myosin light chain kinase [50] (Sigma, 0.1 μM) prevented the appearance of stress fibers observed during reperfusion (**Figure 4**).

3.5 Evidence for alterations of permeability characteristics

Cultured cerebral capillary endothelial cells were incubated in transwells under conditions simulating ischemia (90 minutes) and reperfusion (30 minutes), and permeability properties of the endothelial barrier were measured by quantifying unidirectional flux of ¹⁴C-sucrose across the endothelium [42]. **Figure 5** shows that selective inhibition of Na/H exchange, a brain capillary endothelial carrier shown earlier to contribute to calcium uptake (**Figure 3**) and structural damage (**Figure 4**) during reperfusion, caused a significant reduction in the observed permeability to sucrose.

3.6 Evidence for mitochondrial dysfunction

Figure 6 shows that 1 μM Cyclosporin A (CsA), an inhibitor of mitochondrial damage and apoptosis [42, 43, 51], significantly reduced sucrose permeability across

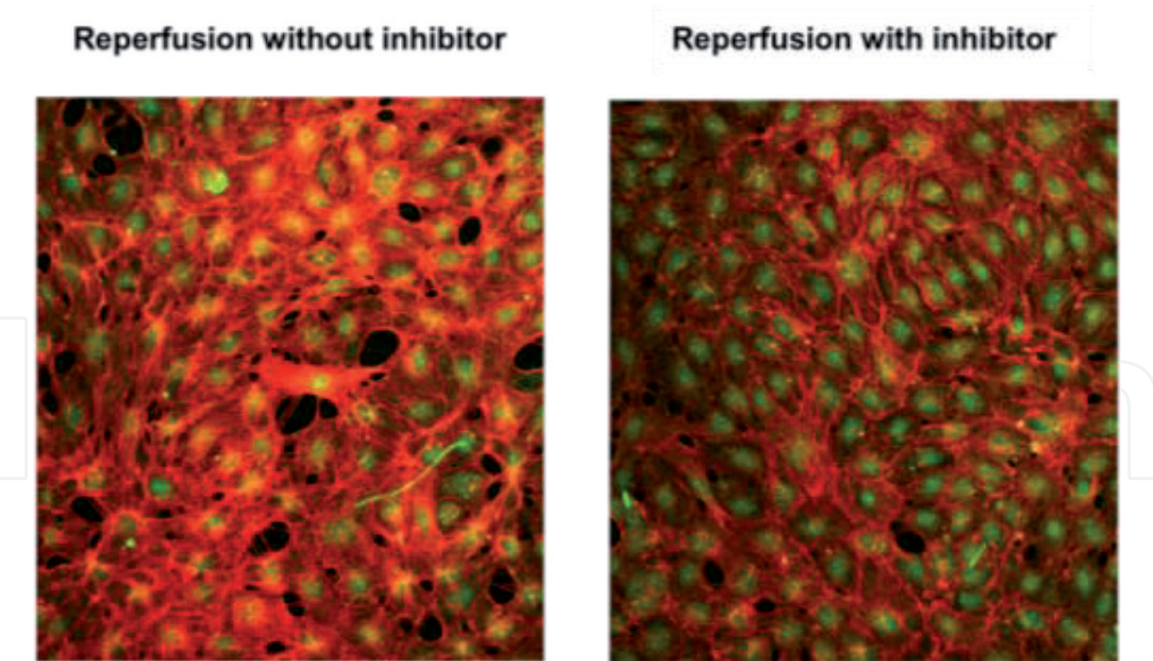


Figure 4. Cultured blood-brain barrier cells were stained for actin with phalloidin following incubation under conditions of ischemia and reperfusion. Normally the actin forms a concentrated ribbon inside the plasma membrane, and ischemia alone causes only minor changes, if any. Simulated reperfusion (30 minutes) following ischemia (90 minutes), however, causes a random and diffuse rearrangement of actin, termed stress fibers (reperfusion without inhibitor). This pattern is typical of cell damage and enhanced permeability of passive solute markers through tight junctions. An inhibitor of calcium-activated myosin light chain kinase (sigma, 0.1 μ M) completely prevented the appearance of stress fibers (reperfusion with inhibitor). These are representative fields from 4 sets of monolayers for each treatment.

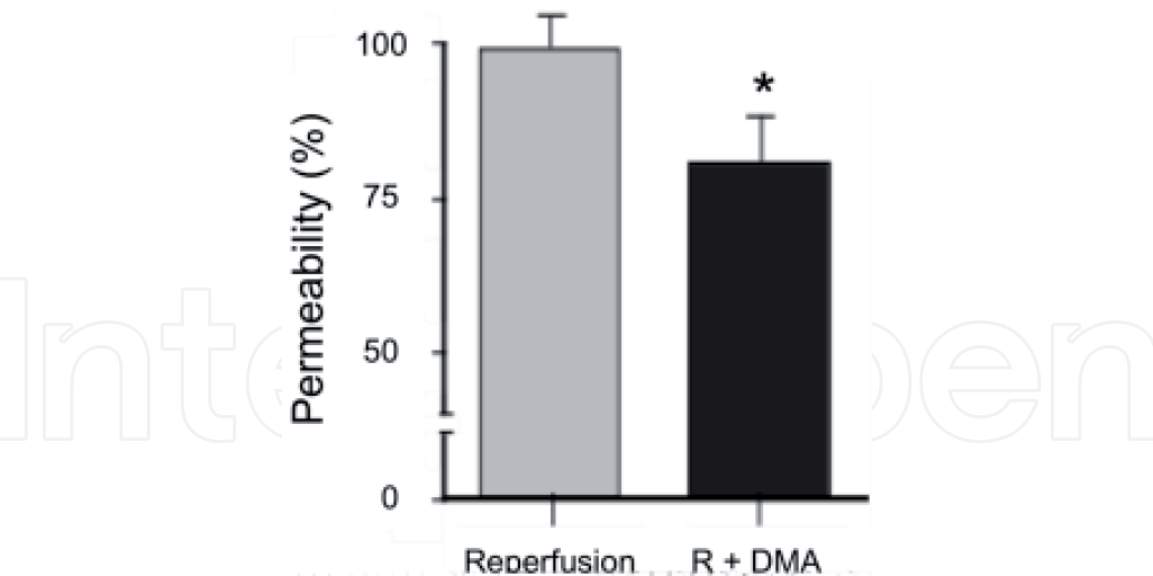


Figure 5. An inhibitor of Na/H antiport (100 μ M DMA, R + DMA) significantly reduced the permeability (% of control) to 14 C-sucrose across cultured brain capillary endothelial cells observed during 90 minutes ischemia/30 minutes reperfusion (reperfusion). *P = 0.05. Values are mean \pm SD, n = 4 observations.

brain capillary endothelial cell monolayers associated with simulated ischemia (90 minutes) and reperfusion (30 minutes). In addition, **Figure 7** confirms that ischemia (30 minutes) followed by reperfusion (24 hours) greatly increased caspase 3 activity that is typically associated with apoptosis [51], and that this was significantly reduced with DMA (100 μ M), an inhibitor of Na/H antiport and calcium loading (**Figure 3**). A specific inhibitor of caspase 3 activity (z-VAD-FMK, 10 μ M)

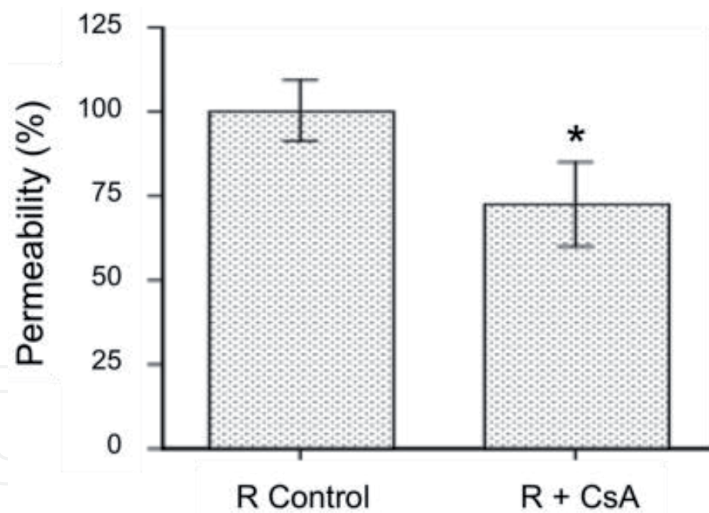


Figure 6. Inhibiting the mitochondrial permeability transition with Cyclosporin A (1 μ M, R + CsA) significantly inhibited the sucrose permeability (% of control) observed during 30 minutes of reperfusion (R control), following 90 minutes of ischemia. * $P < 0.05$. Values are mean \pm SD, $n = 3$ observations.

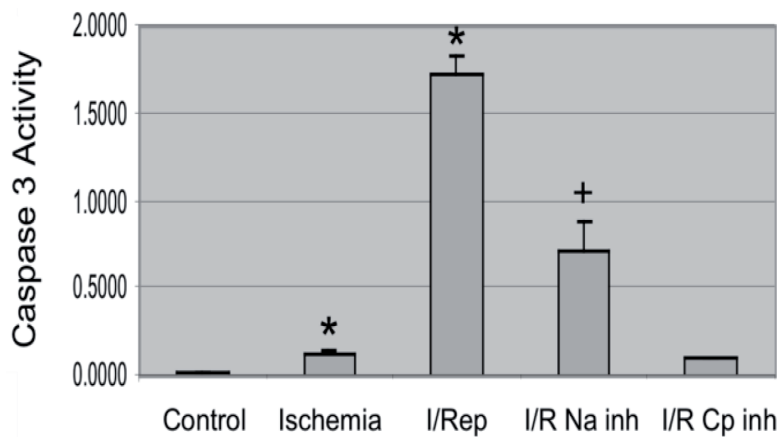


Figure 7. Caspase 3 activity was expressed in cultured blood-brain barrier cells exposed to conditions simulating ischemia-reperfusion. Twenty-four hours of reperfusion following 30 minutes of ischemia resulted in a large increase in caspase 3 activity that was inhibited with DMA (100 μ M). * $P < 0.05$ from control; + $P < 0.05$ from I/rep. I/rep is ischemia plus reperfusion; I/R Na inh is ischemia-reperfusion with DMA inhibition; I/R Cp inh is ischemia-reperfusion with a specific caspase inhibitor. Values are mean \pm SD, $n = 3$ observations.

served as an internal control. The non-mitochondrial caspase 8 pathway for apoptosis showed no significant response in these cells.

3.7 Evidence for the protective role of antioxidants

We have previously shown that the antioxidant γ -glutamylcysteine reduces injury to cultured brain capillary endothelial cells [58] under conditions of simulated ischemia/reperfusion. Cells were incubated for 1.5 hours under ischemic conditions, followed by 3 hours of simulated reperfusion. The presence of 1 mM γ -glutamylcysteine significantly inhibited release of lactate dehydrogenase (LDH) into the incubation medium, thus reducing cell lysis. Additional new studies in our laboratory confirm that the antioxidants glutathione and *N*-acetylcysteine significantly ($P < 0.05$) inhibit LDH release from cultured brain capillary endothelial cells under the same circumstances. Compared to cultured cells incubated under conditions simulating ischemia (1.5 hours) and reperfusion (3 hours) in the *absence* of these antioxidants, 1 mM glutathione and 1 mM *N*-acetylcysteine inhibited mean LDH release by factors of 0.51 and 0.45, respectively. Collectively, the data

indicate that injury to cultured brain capillary endothelial cells exposed to ischemia/reperfusion is significantly reduced in the presence of known antioxidants [19, 20, 45] including native glutathione, and its precursors *N*-acetylcysteine, and γ -glutamylcysteine.

3.8 *In vivo* evidence for a strategy to prevent reperfusion injury

The purpose of the following experiments was to confirm that the mechanisms revealed by our cellular and molecular studies could be used to design a therapeutic approach in whole animals for the treatment of reperfusion injury to the brain capillaries, following transient ischemic stroke. The *in vitro* data suggested that two key factors are responsible for cellular injury and disruption of brain capillaries: (1) a rise in intracellular calcium due to reverse movement of the Na/Ca exchanger during reperfusion, and (2) depletion of endogenous antioxidant activity during the prolonged ischemia, resulting in an elevation of reactive oxygen species upon reperfusion. This led to our conclusion that a reasonable treatment for damage to brain capillary endothelial cells following transient ischemia would be to administer an inhibitor of reverse movement of Na/Ca exchange to prevent the rise in intracellular calcium, and to buffer reactive oxygen species by restoring antioxidant activity within the cells.

To test this hypothesis, middle cerebral artery occlusion (MCAO) was performed on Long-Evans female rats, involving 1 hour of ischemia to the left cerebral hemisphere, and 24 hours of reperfusion. One group of animals received KB-R7943 (10 mg/kg) and γ -glutamylcysteine (400 mg/kg) in 1 ml of isotonic saline solution 1 minute prior to reperfusion (IV, femoral vein), while the other group was administered a placebo (isotonic saline). Following treatment, tissue from the lateral cortex was prepared for electron microscopy, and the cross-sectional area of mitochondria in blood-brain barrier endothelial cells was quantified using morphometric techniques. The contralateral (right) hemisphere served as an internal control for non-ischemic tissue. The data is represented by electron micrographs in **Figure 8** from 2 of 4 animals in each group. As observed in the left panels of **Figure 8** depicting tissue exposed to ischemia/reperfusion without the drugs, mitochondria of blood-brain barrier endothelial cells (solid arrows) are noticeably swollen and abnormal in appearance, indicative of the mitochondrial permeability transition associated with apoptosis [43, 51]. In some instances, mitochondria have actually been extruded into the lumen (dashed arrows) of the capillary, indicating extensive cell damage. In the right panels showing tissue from 2 of 4 stroked animals administered the drugs, the mitochondria are normal, and the blood-brain barrier endothelial cells look healthy. When comparing the groups of animals, morphometric measurements of endothelial mitochondrial size revealed a highly significant ($P = 0.0015$) increase in swelling in stroked animals without the drugs vs. those with the drugs, expressed as a percent change in cross-sectional area from the control contralateral hemisphere (67 ± 15 vs. 13 ± 12 , mean \pm SD, $n = 4$ animals per group).

3.9 *In vivo* evidence for inhibition of apoptosis

Cerebral cortical tissue was randomly sampled from the animals above (1 hour ischemia/24 hours reperfusion), and the TUNEL assay was performed to determine if apoptosis was occurring. Representative tissue from 2 of 4 animals in each group is shown in **Figure 9**. The left panels show cerebral tissue from stroked animals without the drugs. Comparable regions are shown in the right panels, representing tissue from stroked animals that also received the drugs just prior to reperfusion. The cerebral tissue from stroked animals without the drugs showed prominent

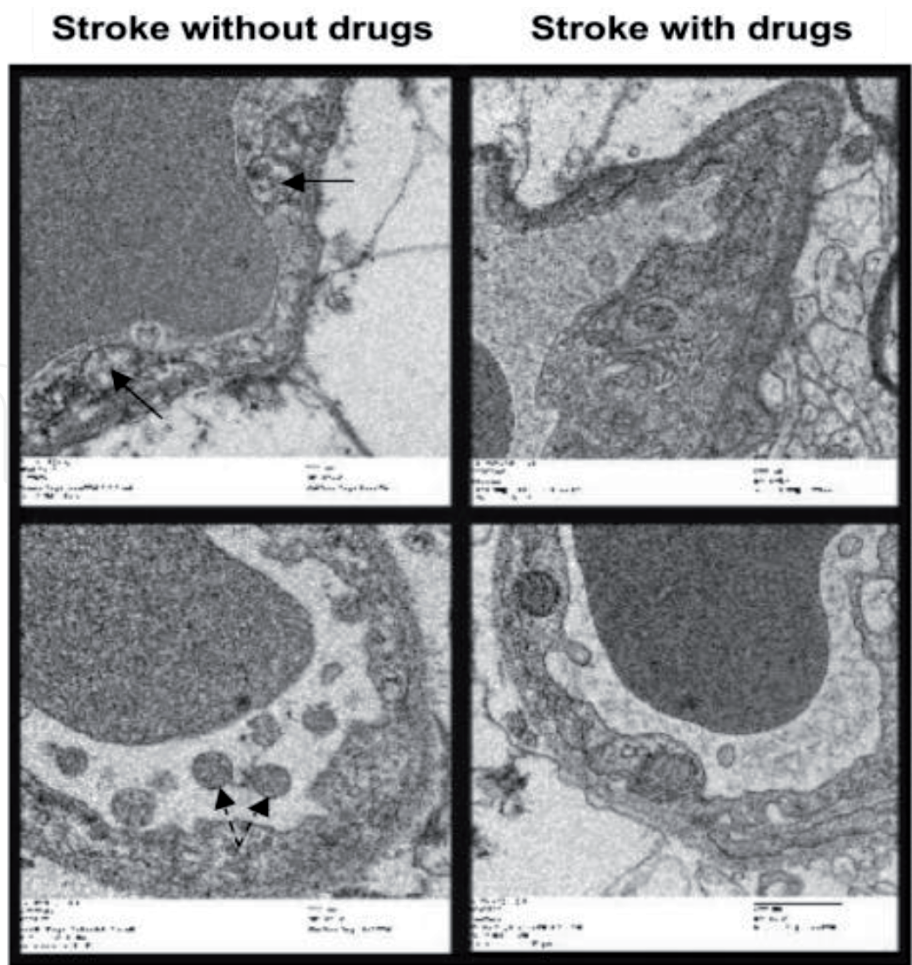


Figure 8. Rats were subjected to conditions of transient ischemic stroke using middle cerebral artery occlusion for 1 hour, followed by 24 hours of reperfusion. One group of stroked animals received cytoprotective drugs (i.e., KB-R 7943 [10 mg/kg] and γ -glutamylcysteine [400 mg/kg]) 1 minute prior to reperfusion, and a second group of stroked animals were administered a placebo. Lateral cerebral cortical tissue was prepared for electron microscopy, and the cross-sectional area of mitochondria in cerebral capillary endothelial cells was measured morphometrically. Two of four stroked animals without the drugs are shown in the left panels of the figure, and two of four stroked animals receiving the drugs are shown in the right panels. It is apparent that mitochondria are swollen in cerebral capillary endothelial cells of stroked animals without the drugs (left panels, solid arrows), suggesting the mitochondrial permeability transition [43] associated with apoptosis [51]. In some cases, damaged mitochondria have been extruded into the capillary lumen (left panels, dashed arrows). By contrast, the mitochondria of stroked animals given the drugs appear normal (right panels). When compared to mitochondria of the unstroked contralateral hemisphere (internal control), the percent increase in cross-sectional area was significantly ($P = 0.0015$) greater for the stroked animals not given the drugs (67 ± 15 vs. 13 ± 12 , mean \pm SD, $n = 4$ animals per group).

nuclear staining indicative of apoptosis, both in cerebral capillaries (arrows) and neural tissue. A morphometric analysis of the number of stained nuclei per unit area for 4 animals in each group (without drugs vs. with drugs) showed a significant difference (3.16 ± 2.00 grains/ mm^2 vs. 0.39 ± 0.47 grains/ mm^2 , $P = 0.036$). No staining was observed in tissue from the contralateral, unstroked cerebral hemispheres.

3.10 *In vivo* evidence for inhibition of neurological dysfunction

To examine the effects of the drugs on neurological behavior following ischemic stroke, the animals for each group above were observed following 1 hour of ischemia and 24 hours of reperfusion. **Table 1** indicates that all 4 of the stroked animals without the drugs showed neurological deficits. Two additional stroked animals not receiving the drugs died. By contrast, 3 of 4 stroked animals that were given the drugs at the time of reperfusion showed no signs of neurological deficit. Furthermore, none of the animals receiving the drugs died.

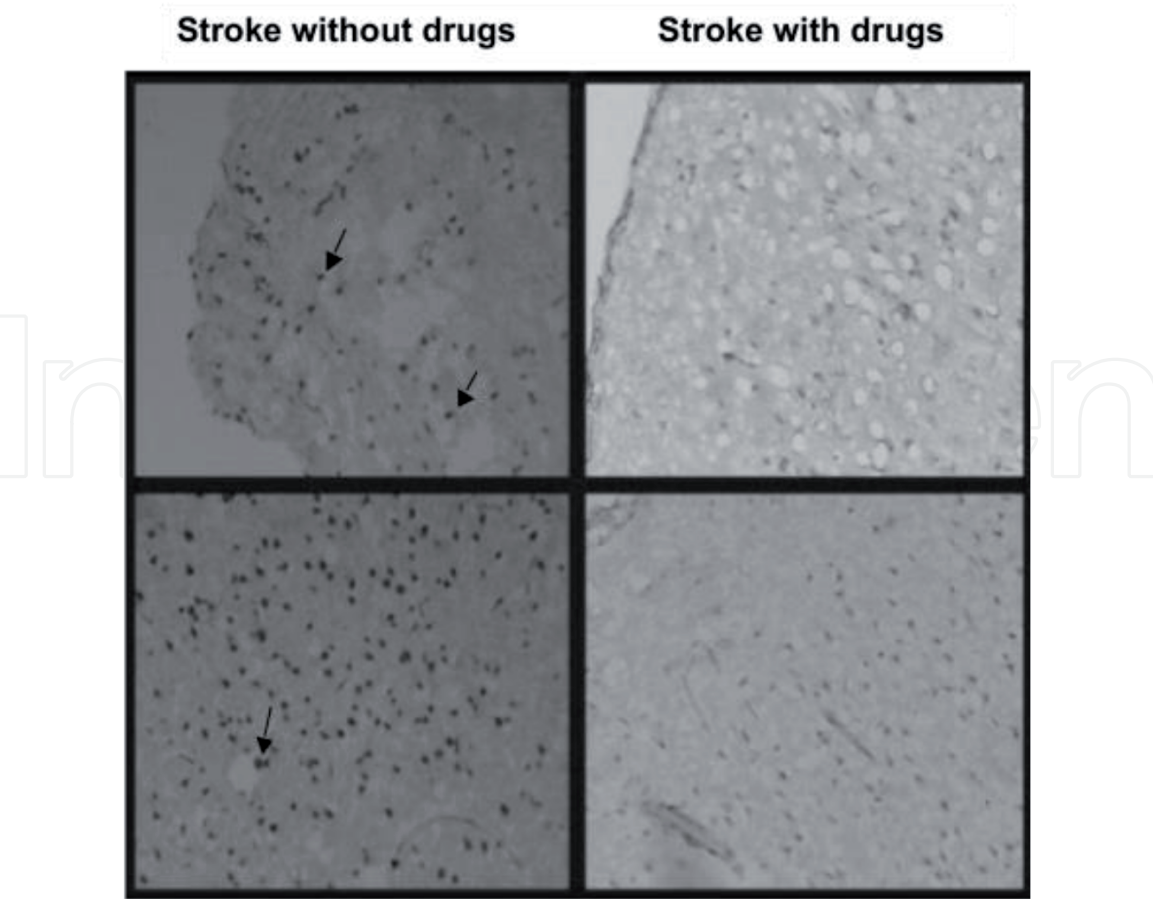


Figure 9. Brain tissue from the above rats subjected to transient ischemic stroke (1 hour ischemia/24 hours reperfusion) was probed for evidence of apoptosis, using the TUNEL assay. Two of four stroke animals without and with the cytoprotective drugs (left and right panels, respectively) are depicted. In this figure, immunocytochemical nuclear reactions indicative of apoptosis are clearly visible in the animals without the drugs (left panels), but are not observed in the animals that were given the drugs (right panels). All four stroked animals without the drugs demonstrated apoptosis in blood-brain barrier endothelial cells (arrows) and neural tissue. Quantifying the number of stained nuclei per unit area showed a significant difference between animals without and with the drugs (3.16 ± 2.00 grains/mm² vs. 0.39 ± 0.47 grains/mm², mean \pm SD, $n = 4$ animals per group, $P = 0.036$).

Stroked animals without drugs:	Stroked animals with drugs:
1. Right front paw deficit	1. No observable deficits
2. Slow moving, with some torticollis	2. No observable deficits
3. Obvious paresis	3. No observable deficits
4. Obvious paresis	4. Displays some motor deficits
Two additional animals died following stroke.	No deaths following stroke.

Table 1. Neurological behavior following transient ischemic stroke.

4. Discussion

The purpose of this study was to define cellular mechanisms that contribute to reperfusion injury of blood vessels within the brain following thrombolysis for ischemic stroke, and to identify pharmacological agents that may be used to prevent cerebral bleeding associated with thrombolytic treatment for stroke. It is known that administering the thrombolytic agent tPA after approximately 3–4.5 hours of ischemia may cause reperfusion injury to brain capillaries [9–11] that can result in cerebral hemorrhage and death [12, 13]. Since approximately 95% of patients with ischemic strokes do not reach the hospital in time to be properly evaluated and

safely administered a thrombolytic agent [18], the possibility of cerebral hemorrhage severely limits the treatment of stroke with tPA.

Cerebral capillary endothelial cells represent the fundamental structure of the blood-brain barrier [52]. These cells are polarized, containing both luminal (blood-facing) and abluminal (brain-facing) plasma membranes with distinct properties [21–23, 25, 26, 53–56]. The cells are held together by tight junctions that effectively inhibit paracellular transport [21], implying that the properties of the respective luminal and abluminal plasma membranes regulate transport across the blood-brain barrier. Based upon observations in other tissues [40, 57], we hypothesized that ischemia-reperfusion injury to cerebral capillary endothelial cells is due to oxidative injury associated with the formation of reactive oxygen species in the presence of elevated intracellular calcium during reperfusion [58–62]. This would require loss of intracellular antioxidant (e.g., glutathione) during prolonged ischemia, formation of free reactive oxygen species and uptake of calcium during re-oxygenation, and damage to mitochondria causing programmed cell death (apoptosis).

To test this hypothesis, we began by determining if the cellular processes required for these mechanisms are functional in blood-brain barrier endothelial cells. We reasoned that intracellular glutathione, an endogenous antioxidant, would diffuse out of the cells during ischemia, utilizing passive carriers we had previously described on both plasma membranes [58]. We next provided evidence that both Na/H (NHE1) and Na/Ca (NCX 1) exchangers are present on the plasma membrane domains of these cells, and that conditions simulating ischemia and reperfusion result in elevated intracellular calcium due to activation of sodium-hydrogen ion antiport and reverse movement of sodium-calcium exchange (**Figure 3**). Cellular lysis following these events was significantly inhibited by adding the antioxidant γ Glu-Cys [19, 20, 58], which is consistent with the formation of reactive oxygen species expected upon re-oxygenation in glutathione depleted cells. These findings reinforced our interpretation that a combination of elevated intracellular calcium and reactive oxygen species are involved in injury to cerebral capillary endothelial cells, under conditions of ischemia and reperfusion.

Our studies further indicated that injury to brain capillaries involves two phases. An early increase in permeability to sucrose (**Figure 5**), a marker of paracellular transport, was associated with the formation of actin stress fibers inside the endothelial cells (**Figure 4**). This was, in turn, accompanied by the appearance of large intercellular holes (**Figure 4**), apparently due to the opening of tight junctions [63]. We reasoned that such a change could be due to activation of myosin light chain kinase in the presence of the elevated intracellular calcium that we previously observed (**Figure 3**). Since it has been shown that ischemia causes actin filaments to conjugate with ZO-1 [64], a tight junctional protein, force generated by contraction of the cytoskeleton could weaken the tight junctions and result in the formation of stress fibers. Indeed, treatment with a myosin light chain kinase inhibitor effectively reversed the effects of simulated ischemia and reperfusion on the formation of actin stress fibers, and the appearance of large intercellular holes (**Figure 4**). These morphological and functional changes in cerebral capillary endothelial cells occurred within a few hours of exposure to conditions simulating ischemia and reperfusion, and generally correlated with an early and reversible phase of altered permeability to the cerebral vasculature when exposed to conditions of ischemia and reperfusion *in vivo* [64].

A second phase of injury to cerebral capillaries following transient ischemia and reperfusion involved apoptosis and endothelial cell death. In an initial set of experiments, sucrose permeability was measured across monolayers of cultured

blood-brain barrier endothelial cells exposed to conditions simulating ischemia and reperfusion. Permeability was significantly reduced by co-incubating with cyclosporin A (**Figure 6**), an inhibitor of the mitochondrial permeability transition associated with the early stages of apoptosis [42, 43, 51]. Under similar conditions, cultured blood-brain barrier endothelial cells expressed a large increase in caspase 3 activity after 24 hours of simulated reperfusion following ischemia (**Figure 7**), indicating activation of the apoptotic pathway [51].

These *in vitro* findings were consistent with our working hypothesis which predicted that elevated intracellular calcium and reactive oxygen species would activate apoptosis in cerebral capillary endothelial cells exposed to conditions of ischemia and reperfusion. Furthermore, the results suggested that: (1) inhibiting reverse movement of Na/Ca exchange with KB-R7943 [65], and (2) replenishing lost antioxidant with γ Glu-Cys [19, 20] would prevent reperfusion (oxidative) injury to brain capillaries. Since γ Glu-Cys is a precursor of the antioxidant glutathione [59] lost during ischemia, and γ Glu-Cys itself possesses antioxidant properties [19, 20], it represents a reasonable antioxidant therapeutic in this setting.

The next logical step was to determine if drugs that prevent increased levels of intracellular calcium and reactive oxygen species in brain capillary endothelial cells would inhibit damage to cerebral capillaries *in vivo*. Thus, rats were exposed to middle cerebral artery occlusion to simulate ischemic stroke, after which the animals were either treated with a placebo (isotonic saline), or administered a combination of γ Glu-Cys (400 mg/kg) and KB-R7943 (10 mg/kg) in isotonic saline that was infused intravenously approximately 1 minute prior to initiating reperfusion of cerebral blood flow. The rationale was that administering the combination of drugs immediately before re-establishing cerebral blood flow would protect the endothelial cells of cerebral capillaries from oxidative injury upon reperfusion. **Figure 8** indeed illustrates that such a therapeutic approach significantly inhibited morphological damage to cerebral capillary endothelial cells, including swelling of the mitochondria that is indicative of oxidative injury and the permeability transition that precedes apoptosis [51, 61, 62]. Furthermore, the Tunnel assay revealed that co-infusion of the drugs immediately before reperfusion inhibited the appearance of apoptosis in representative cerebral cortical tissue 24 hours after re-establishing blood flow to the brain (**Figure 9**). Finally, an assessment of neurological behavior confirmed that use of the drugs inhibited functional damage due to ischemia and reperfusion (**Table 1**).

5. Conclusions

Together these studies support the interpretation that prolonged cerebral ischemia followed by reperfusion of oxygenated blood may cause oxidative damage to cerebral capillary endothelial cells consistent with loss of vascular integrity and hemorrhage. The mechanisms involve mitochondrial injury and apoptosis due to the formation of reactive oxygen species in the presence of elevated cellular calcium. This condition may be treated by using the antioxidant γ Glu-Cys to buffer reactive oxygen species, and a Na/Ca inhibitor (reverse mode) to prevent calcium loading in cerebral capillary endothelial cells. The *in vivo* studies indicate that co-administering both drugs intravascularly 1 minute prior to reperfusion provides cytoprotection that reduces reperfusion injury to the cerebral vasculature. Thus, this may represent a means of prolonging the window of opportunity to use a thrombolytic agent for treatment of ischemic stroke, and reduce the occurrence of hemorrhagic transformation in a clinical setting.

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Conflict of interest

Dr. Peterson (inventor) and Rosalind Franklin University of Medicine and Science (assignee) have been awarded patents that are related to research done in this study. They both may benefit financially upon commercialization of the patents. Dr. Peterson is a Professor at Rosalind Franklin University of Medicine and Science and owns Harbor Biotechnology, LLC, a company that seeks to commercialize the patents referenced above. Further details may be obtained from Dr. Peterson upon request.

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