Combination of Hypoxia/Aglycemia Compromises In Vitro Blood-Brain Barrier Integrity

THOMAS J. ABBRUSCATO and THOMAS P. DAVIS

Department of Pharmacology, University of Arizona College of Medicine, Tucson, Arizona

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ABSTRACT

Increased cerebrovascular permeability is an important factor in the development of cerebral edema after stroke, implicating the blood-brain barrier (BBB) in the pathology of stroke. Present investigations modeled stroke at the level of the cerebral capillary endothelium by analyzing BBB permeability changes to the membrane-impermeant marker [14C]sucrose after hypoxia/aglycemia. Under hypoxia alone, long exposures (48 h) were necessary to result in a significant increase in permeability of bovine brain microvessel endothelial cells to [14C]sucrose. Hypoxia/aglycemia exposures resulted in a much shorter time (i.e., 1–3 h) required for a corresponding increase in permeability to [14C]sucrose. Statistically significant changes in basal permeability were observed between 3 and 6 h of hypoxia/aglycemia; however, 6 h of aglycemia alone had no significant effect on BBB permeability. Both rat astroglialoma (C6) cells and C6 conditioned medium showed no improvements in barrier function measured by transendothelial cell resistance or permeability to [14C]sucrose. Changes in endothelial cell calcium flux may be responsible for the permeability change observed after both 48 h of hypoxia and 6 h of hypoxia/aglycemia because nifedipine (10 and 100 nM) and SKF 96385 (100 nM) decreased the permeability change. Immunocytochemical studies also revealed a change in the distribution of endothelial cell F-actin. This study provides evidence that the BBB is sensitive to short exposures of hypoxia/aglycemia and that changes in endothelial cell calcium flux may be responsible for structural and functional variations in the BBB during ischemic stress.

The blood-brain barrier (BBB) is the interface between the blood and central nervous system (CNS) that limits the transit of molecules based on size, charge, hydrophobicity, and/or affinity to carriers. The selective nature of the BBB helps to maintain the homeostasis of the CNS environment to ensure proper brain functioning. The BBB consists of the cerebral capillary endothelium, which contains tight junctions that form rows of extensive, overlapping occlusions that block the intercellular route of solute entry into the CNS (Brightman and Reese, 1969). Damage to the BBB during hypoxia/stroke can result in serious clinical circumstances that influence stroke outcome: vasogenic brain edema and inflammation (Baethmann et al., 1983; Hatashita and Hoff, 1990). Any change in the basal permeability of the cerebral capillary endothelium (paracellular or transcellular) can exacerbate a variety of pathologic processes (Hirano et al., 1994). Increased BBB permeability has been shown to play an important pathophysiological role during posts ischemic reperfusion (Yang and Betz, 1994). Several researchers have shown that ischemia followed by reperfusion results in BBB disruption (Sadoshima et al., 1983; Sage et al., 1984; Dobbins et al., 1989); however, the actual mechanism or biochemical changes that occur in cerebral capillary endothelial cells after ischemic insult have not been fully investigated. The role of the BBB in stroke pathology is an important area because stroke is a leading cause of death and disability for men and women of all ages, socioeconomic classes, and ethnic origins. Much of the current stroke research has extensively focused on the response of neurons and astrocytes to hypoxic or ischemic insults (Yu et al., 1989; Murphy and Horrocks, 1993). Neuronal injury in mouse cortical cultures was observed as early as 8 h of hypoxic exposure (Goldberg et al., 1987), and astrocytic injury was observed some time between 12 and 18 h (Yu et al., 1989). However, little is known about the response of cerebral capillary endothelial cells to hypoxia. Experiments have been performed in vivo where focal ischemia was produced by occluding the left middle cerebral artery in rats (Hatashita and Hoff, 1990). A significant increase in the albumin transfer index was observed within the first 12 h after occlusion.

Endothelial cell damage can occur from oxygen and glucose deprivation after a hypoxic episode induced by stroke. Using...
a cell culture model of the BBB, a researcher can more precisely investigate the mechanisms associated with endothelial cell response to hypoxia and aglycemia. In vitro models of the BBB can be manipulated more precisely to reflect in vivo changes due to stroke, which is classically defined as a “cerebrovascular accident” caused by the reduction of blood supply, resulting in a decrease in oxygen tension and high-energy metabolites (glucose) in areas of the brain.

Calcium pools have been implicated in the maintenance of the characteristic tight junction structure across the cerebral endothelium (Joo et al., 1992). Changes in endothelial calcium may be the cause of permeability changes at the BBB. The present research will attempt to define the role of endothelial cell calcium in BBB disruption during hypoxia/aglycemia. It is possible that the changes in BBB permeability could be due to microtubular rearrangements stimulated by changes in calcium flux. Microtubules have been shown to depolymerize at high calcium ion concentrations, and endothelial actin filaments have been shown to be important for maintaining BBB permeability to protein (Nag, 1995).

In the present study, we used a cell culture model of the BBB consisting of primary cultured bovine brain microvessel endothelial cells (BBMECs) to define the time course with which hypoxia and aglycemia alter endothelial cell permeability. Experiments were performed by coculturing rat astroglia (C6) cells with BBMECs to determine whether this model required any astroglia influence to produce a more restrictive endothelium. In addition, we pharmacologically manipulated this model to determine whether the damage is due to changes in intraendothelial cell calcium by using both inhibitors of voltage-regulated calcium channels and capacitative calcium entry. Cells were treated with hypoxia (95% N₂ and 5% CO₂) according to the method previously described by Yu et al. (1989) and Gobbel et al. (1994), in which the concentration of oxygen in the atmosphere was maintained at 0% oxygen and the PO₂ in the medium was below 25 mm Hg. We chose to use both hypoxic and aglycemic conditions to more precisely reflect the changes that occur during cerebral ischemia where glucose and oxygen are reduced in capillary beds.

**Materials and Methods**

**Radiolabeled Compounds.** [3H]-[α-Pen²,β-pen⁵]-enkephalin (DPDPE) (42 Ci/mmol) was obtained from Chirone Peptide Systems (San Diego, CA) under the direction of the National Institute of Drug Abuse. [14C]Sucrose (672 mCi/mmol) was purchased from New England Nuclear Research Products (Boston, MA). [125I]Insulin (2200 Ci/mmol) was purchased from Diagnostic Products Corporation (Los Angeles, CA).

**In Vitro BBMEC Model.** Fresh bovine brains were obtained from a local slaughterhouse. BBMECs were isolated from the gray matter of the cerebral cortex and cryopreserved, as previously described (Audus and Borchardt, 1986, 1987). The isolated cells were seeded at a cell density of 50,000 cells/cm² onto tissue culture dishes that had been precoated with rat tail collagen and fibronectin and contained 25-mm Costar polycarbonate membrane filters (Costar Nucleopore, Cambridge, MA) with a 10-μm pore size. After the spindle-shaped cells were grown to confluence (10–12 days), the BBMEC monolayers were chosen because they have been shown to improve barrier integrity in BBMECs (Raub et al., 1992). Confluent BBMEC monolayers were achieved using the equation: PC = flux/(A*Cp), where flux is the slope of the line, A is the area of the membrane (0.636 cm²), and Cp is the initial donor concentration. The donor concentration did not change by more than 10% throughout experiments. A single monolayer was used for each experimental n number.

**Resistance Measurements.** Endothelial cell monolayer health and confluence was monitored using a Millicell ERS device (Millipore, Bedford, MA). The extracellular matrix-treated transwell insert was placed in a sterile 24-well plate containing culture medium and used to measure background resistance. The resistances of these blank filters were then subtracted from those of filters with cells before final resistances were calculated (Ω·cm²). Confluent monolayers were achieved by day 12 and subsequently used for permeability studies above.

**C6 Coculture and C6 Conditioned Medium.** C6 cells were obtained from American Type Culture Collection (CCL-107; Rockville, MD) and consisted of a transformed rat glioma cell line that has been shown to induce BBB characteristics on a variety of endothelial cells. BBMECs were grown as described on transwell filter inserts with a 0.4-μM pore (Costar, Cambridge, MA). At day 10, each transwell insert along with BBMECs was transferred to a well that had either BBMEC culture medium, C6 conditioned medium (CM), or C6 cells (40,000 cells/cm²) that had been growing for 1 day in the lower, basolateral chamber. C6 cells were grown in BBMEC growth medium without heparin and supplemented with 2% (v/v) FBS. CM was produced by plating C6 cells on the bottom of 24-well plates at 40,000 cells/cm². Medium was collected every 3 days. This medium was then diluted with an equal volume of fresh C6 medium. These conditions were chosen because they have been shown to improve barrier integrity in BBMECs (Raub et al., 1992). Confluent BBMEC monolayers were used for transendothelial permeability experiments at day 12. PCs for [14C]sucrose were calculated as described above by sampling the radioactivity entering into the basolateral well after 60 min. These permeability experiments were performed on a rotating table that was placed in a humidified, 37°C incubator.

**BBMEC Uptake Studies.** BBMECs were grown to confluence on 24-well plates precoated with rat tail collagen and human fibronectin. Growth medium was removed, and the cells were preincubated in PBS. After 20 min. 0.33 μCi of either [14C]sucrose, [3H]DPDPE, or [125I]insulin was added to each well. The cells were then incubated for 20 min at 37°C on a shaker table. The radioactive buffer was...
removed, and the cells were washed three times with ice-cold assay buffer. Cells were then incubated with 1 ml of 1% Triton X-100 for 30 min. A portion of the Triton X-100 sample was prepared for radio- active sampling as described above. The other portion of sample was assayed for protein using a Pierce BCA protein kit. Unidirectional rate constants (K_{cell}; μL·min^{-1}·mg^{-1}) (Begley et al., 1996) were determined by single-time point analysis and normalized for protein content: K_{cell} = C_{buffer}/C_{cell} × T, where C_{buffer} is the concentration of drug in the buffer and C_{cell} is the concentration of drug in the cell at time T in minutes.

**Hypoxia/Aglycemia Treatment.** Confluent monolayers of BBMECs were exposed to hypoxic or hypoxic/aglycemic conditions by adding either complete medium or RPMI 1640 (without L-glucose) bubbled with 95% N2/5% CO2 at 3 liters/min for 5 min. Hypoxia was induced by placing cells in a humidified, sealed chamber (Billups-Rothenberg, Del Mar, CA) at 37°C that had been flushed with 95% N2/5% CO2 (Yu et al., 1989; Gobbel et al., 1994). For longer hypoxic exposure times, the modular incubator chambers were flushed with the 95% N2/5% CO2 gas mixture for 15 min every 12 h at a flow rate of 3 liters/min.

**HPLC Analysis of [3H]DPDPE and [125I]Insulin Stability.** Aliquots from the receiver chamber were taken after 120 min of BBMEC exposure. Stability of drugs was determined as previously described (Abbruscato et al., 1997b). Samples were analyzed using a series 410 HPLC gradient system (Perkin-Elmer Inc., Norwalk, CT). Samples were eluted from a Beckman Instruments Inc. Ultrasound column (4.6 × 250 mm) with a curvilinear gradient of 20 to 50% of 0.1% trifluoroacetic acid in acetonitrile versus 0.1% aqueous trifluoroacetic acid over 30 min at a flow rate of 1.5 ml/min and a column temperature of 37°C. After separation on the HPLC column, the outflow was routed to the online A200 Flo-One Radioactive Detector equipped with a 2.5-ml flow cell (Packard Radiomatic Instruments, Meriden, CT) for real-time HPLC analyses of eluates.

**BBMEC Cytoskeletal Distribution of F-Actin.** Staining of the F-actin cytoskeleton was performed by growing BBMECs to confluence (12 days) on glass coverslips precoated with rat tail collagen. Confluent monolayers of BBMECs were washed three times in PBS, fixed with 4% paraformaldehyde, and then washed again with 25 mM glycine buffer. Cells were permeabilized with 0.1% Triton X-100 for 10 min and washed for 5 min with PBS. Fluorescein isothiocyanate (FITC)-phalloidin (0.1 mg/ml) (Sigma Chemical Co., St. Louis, MO) was used as a fluorescent phallotoxin to identify filamentous actin at 200-, 300-, and 500-fold dilutions. Imaging was accomplished by using a standard inverted wide-field microscope designed for epifluorescence (Zeiss IM-35) with an Olympus S Plano Apochromatic 60 × 1.41 NA objective, oil emission lens, excitation wavelength of 485 nm, and emission filter of 525 nm coupled to a slow scanning CCD camera. Image processing was accomplished using PMIS imaging software.

**Statistical Analysis.** For all experiments, the data are presented as the mean ± S.E.M. Student’s t test was used for the comparison of two mean values, and statistical significance was taken as *p < .05 and **p < .01.

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**Results**

Normoxic and Hypoxic BBMEC Permeability to [14C]Sucrose. The permeability of the BBMECs to the impermeable marker [14C]sucrose was studied under normoxia and after 24- and 48-h hypoxia exposures. Twenty-four hours of hypoxia had no significant effect on basal BBB permeability compared with the cells grown in normoxic conditions, although 48 h of hypoxia did result in a statistically significant increase in the basal permeability at all time points as shown in Fig. 1 (P < .01).

**Permeability of Hypoxic/Aglycemic BBMECs to [14C]Sucrose.** One hour of hypoxic/aglycemic conditions resulted in a small but not statistically significant change in the permeability to [14C]sucrose compared with normoxic conditions. However, 3 h of hypoxia/aglycemia resulted in a significant increase in permeability to [14C]sucrose (P < .05). Longer hypoxic/aglycemic exposures (6, 12, 24, and 48 h) resulted in statistically significant increases (P < .01) at all time points studied (Fig. 2). Six hours of aglycemia alone had no significant effect on the permeability to [14C]sucrose compared with normoxia.

**Resistance Measurements.** Transendothelial cell resistance (TEER; Ω cm²) was consistently observed to increase from day 6 to day 10. The addition of either C6 cells or C6 CM showed no effect on the TEER from day 10 to day 12 (Fig. 3).

**Effect of C6 Gliomas and C6 CM on BBMEC Permeability to [14C]Sucrose.** The permeability of the BBMECs to [14C]sucrose was found to be statistically increased after 6 h of hypoxia/aglycemia treatment for endothelial cells cul-

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**Fig. 1.** PC (cm/min × 10⁻⁶) of [14C]sucrose after 24 and 48 h of hypoxia. Data are expressed as mean PC ± S.E.M. at each time point sampled. Statistical significance was compared with the PC of [14C]sucrose crossing normoxic BBMEC monolayers.

**Fig. 2.** PC (cm/min × 10⁻⁶) of [14C]sucrose across hypoxic/aglycemic BBMECs. Data are expressed as mean PC ± S.E.M. at each time point sampled. Statistical significance was compared with the PC of [14C]sucrose crossing normoxic BBMEC monolayers.
tured alone \((P < .01)\), with C6 CM \((P < .05)\) or with C6 coculture \((P < .01)\) (Fig. 4). C6 CM and C6 coculture had no statistically significant effect on normoxic \(^{14}\text{C}\)sucrose permeability.

Nifedipine Treatment With 48 h of Hypoxia and 6 h of Hypoxia/Agylycemia. The permeability of BBMECs to \(^{14}\text{C}\)sucrose after 48 h of hypoxia was measured in the presence of the L-type calcium antagonist nifedipine (1–100 nM) as shown in Fig. 5. Nifedipine (10 nM) significantly reduced the permeability change induced after 48 h of hypoxia \((P < .05)\). The highest concentration of nifedipine (100 nM) resulted in a significant decrease in the permeability change induced by 48 h of hypoxia \((P < .05)\) but was not significantly different than treatment with 10 nM nifedipine at any time point. The lowest concentration of nifedipine (1 nM) showed no statistical difference compared with the 48-h hypoxia treatment. Nifedipine (10 nM) significantly reduced the permeability change induced by 6 h of hypoxia/agylycemia \((P < .01)\) (Fig. 5). Control experiments were performed showing that 100 nM nifedipine had no affect on baseline \(^{14}\text{C}\)sucrose permeability (data not shown).

SKF 96365 Treatment With 6 h of Hypoxia/Agylycemia. BBMECs treated with 100 nM SKF96365 showed a similar reduced permeability change to \(^{14}\text{C}\)sucrose after 6 h of hypoxia/agylycemia that was statistically significant \((P < .05)\) (Fig. 6). SKF96365 (100 nM) was shown to have no effect on baseline \(^{14}\text{C}\)sucrose permeability (data not shown).

\([^{3}\text{H}]\text{DPDPE and } [^{125}\text{I}]\text{Insulin Transport Across BBMECs After 48-h Hypoxia.}\) Transport of the modified enkephalin \([^{3}\text{H}]\text{DPDPE} \) was found to be significantly reduced \((P < .05)\) (Fig. 7a). \([^{125}\text{I}]\text{Insulin transport across the BBMECs after 48 h of hypoxia was not statistically different at all time points compared with normoxic BBMEC transport of } [^{125}\text{I}]\text{insulin} \) (Fig. 7b).

BBMEC Uptake of \(^{14}\text{C}\)Sucrose, \([^{3}\text{H}]\text{DPDPE, and } [^{125}\text{I}]\text{Insulin.}\) The uptake of either \(^{14}\text{C}\)sucrose and \([^{125}\text{I}]\text{insulin} \)
Fig. 7. PC (cm/min × 10⁻⁶) of (a) [³H]DPDPE and (b) [¹²⁵I]insulin after 48 h of hypoxia. [¹⁴C]Sucrose permeability has been subtracted from the total PC of each compound. [³H]DPDPE transport is significantly reduced after 48 h of hypoxia (*significance at P < .05 using Student’s t test). Data are expressed as mean PC ± S.E.M. (n = 3–4 BBMEC monolayers).

Stability of [³H]DPDPE and [¹²⁵I]Insulin After 120 min in BBMECs. HPLC revealed that [³H]DPDPE and [¹²⁵I]insulin samples from the receiver chamber were >90% intact after a 120-min BBMCE passage (data not shown). This was accomplished by direct injection of receiver chamber samples on the HPLC column for real-time analysis.

Table 1

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<th>6-h hypoxic/aglycemic</th>
<th>Normoxic</th>
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<tr>
<td>[¹⁴C]Sucrose</td>
<td>0.66 ± 0.18[₈]</td>
<td>0.61 ± 0.16</td>
</tr>
<tr>
<td>[³H]DPDPE</td>
<td>1.94 ± 0.13[₈]</td>
<td>1.30 ± 0.21</td>
</tr>
<tr>
<td>[¹²⁵I]Insulin</td>
<td>1.78 ± 0.11[₈]</td>
<td>1.86 ± 0.16</td>
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[₈] Significance at P < .05 compared with normoxic uptake. n = 6–8 wells/treatment.

Discussion

The aim of the present study was to examine the effects of hypoxia/aglycemia on the permeability characteristics of BBB endothelial cells. The BBMEC model provides an excellent barrier preventing the entry of hydrophilic compounds. Under normoxic conditions, the BBMEC model shows restricted permeability to [¹⁴C]sucrose with values similar to that reported by Dehouck et al. (1992) (6.3 × 10⁻⁶ cm/min), who used bovine brain capillary endothelial cells with an astrocytic coculture. TEER was measured to determine whether the C6 rat glioma coculture or CM would enhance the electrical resistance of the in vitro BBMCE model. It is apparent in Fig. 3 that TEER was not enhanced with either C6 coculture or CM. In fact, TEER values in this study are somewhat higher than that reported by Raub et al. (1992), who used a similar model. This apparent lack of a TEER response of BBMECs to C6 cells or CM may be due to our rapid isolation techniques or species variation in our cell culture system. Endothelial cells used in these studies are derived from fresh bovine brains, and C6 cells consist of a transformed rat glioma. In the literature, it has been shown that CNS tumors have been associated with a relatively leaky BBB (Greig, 1989) and that CNS tumors may secrete a factor (vascular permeability factor) that causes a leaky brain endothelium (Connolly et al., 1989). We also observed no apparent difference in endothelial cell response to hypoxia/aglycemia with either C6 coculture or C6 CM compared with BBMECs cultured alone (Fig. 4). This ensures us that the BBMEC model is suitable for measuring changes in paracellular diffusion.

Initial investigations examined the endothelial cell response to hypoxia under physiological glucose concentrations (10 mM). A dramatic increase in the permeability to [¹⁴C]sucrose was noted after 48 h of hypoxia (Fig. 1). Hypoxic exposures below 24 h had no effect on basal BBB permeability to [¹⁴C]sucrose. These results are in agreement with those of Plateel et al. (1995), who found that barrier function is maintained during the first 24 h of hypoxia but that at 48 h, a dramatic increase in the permeability of the monolayer to sucrose and insulin is observed. Gobbel et al. (1994) also studied the effect of hypoxia on the viability characteristics of in vitro rat brain cerebral endothelial cells. The endothelial cells were observed to be tolerant of up to 24 h of hypoxia as indicated by both lactate dehydrogenase release and ethidium bromide staining. The reason why 48 h of hypoxia alone was necessary for changes in endothelial cell permeability may be related to the metabolic pathways used for energy production by these cells. Although we are producing a severe hypoxia in vitro (medium PO₂ < 25 mm Hg), glucose levels may be maintained longer in our in vitro system compared with in vivo, where aglycemia would occur more readily because of the voracious appetite of the brain for...
glucose. In addition, the transport of glucose may be upregulated to the ischemic core region to maintain neuronal survival. Hypoxia has been shown by two different groups to increase glucose transport and the level of glucose transporter-1 expression at the BBB in rats (Harik et al., 1994; Vannucci et al., 1996). Thus, the observations by us and other researchers that endothelial cells are relatively resistant to hypoxic exposure may be due to the delayed onset of aglycemia compared with that observed during clinical stroke.

To address the possibility of aglycemia potentiating hypoxic injury (BBB opening), we performed experiments under both hypoxic/aglycemic conditions to determine whether the absence of glucose influences the time course of injury. Our data shows that our in vitro endothelial cell model of the BBB is much more sensitive under hypoxic/aglycemic conditions. Three hours of hypoxia/aglycemia resulted in a statistically significant increase in $[^{14}\text{C}]$sucrose permeability ($P < .05$). The increase in $[^{14}\text{C}]$sucrose permeability occurred in a time-dependent fashion at 1 to 24 h. $[^{14}\text{C}]$Sucrose permeability plateaued at 24 to 48 h. It is apparent that removal of glucose from the culture media has a dramatic effect on the sensitivity of cerebral capillary endothelial cells to hypoxic insult and that the time course of injury is dramatically shortened compared with hypoxic exposure alone. Hypoxic damage to endothelial cells may be made worse under aglycemia because of rapid ATP depletion. Thus, hypoxia/aglycemia is a better model of in vivo ischemia where blood flow is blocked and hypoxia and hypoglycemia develop quickly. This research shows that the endothelial cells that make up the BBB may be as sensitive as astrocytes and neurons to hypoxia when an aglycemic environment is introduced into the system.

The role of endothelial cell calcium on the permeability of the BBB was investigated by using the L-type calcium channel antagonist nifedipine at concentrations ranging from 1 to 100 nM. Lower concentrations were chosen so as to maintain the selectivity of this dihydropyridine for calcium channels while minimizing the antioxidant activity of nifedipine that has been previously described (Mak et al., 1995) To our knowledge, it remains debatable whether BBMECs express voltage-regulated Ca$^{2+}$ channels. After a thorough literature review, it appears that BBMECs have not been shown to express L-type Ca$^{2+}$ channels, yet Morel and Godfraind (1990) have shown that brain microvessels isolated from the rat express voltage-dependent, stereoselective binding sites for dihydropyridine calcium antagonists. In addition, Strasser et al. (1997) have shown that rat brain endothelial cells exposed to hypoxia/reoxygenation exhibited significantly less production of reactive oxygen species in the presence of the L-type Ca$^{2+}$ channel antagonist verapamil. Dihydropyridine compounds appear to be acting through a different mechanism in our experiments because brain endothelium presumably is nonexcitable tissue. Nifedipine (10 nM) significantly reduced the permeability change induced by 48 h of hypoxia ($P < .05$) and 6 h of hypoxia/aglycemia ($P < .01$) (Fig. 5). It is apparent that endothelial cell calcium

![Fig. 8. Representative images of the cytoskeletal distribution of F-actin shown by FITC-phalloidin staining. Imaging was accomplished by using a standard inverted wide-field microscope designed for epifluorescence (Zeiss IM-35) with an Olympus S Plan Apochromatic 60 x 1.41 NA objective, oil immersion lens, excitation wavelength of 485 nm, and emission filter of 525 nm coupled to a slow scanning CCD camera. Treatments are 0, 24, and 48 h of hypoxia and 0, 3, and 6 h of hypoxia/aglycemia. Bar, 10 μm.](https://jpet.aspetjournals.org/doi/abs/10.1124/jpet.1999.275.6.673)
flux is occurring during 48 h of hypoxia and 6 h of hypoxia/aglycemia and that this change is influencing BBB integrity.

The idea of calcium influx affecting BBB integrity during hypoxia/aglycemia was also tested using the blocker of the non-specific cation channel SKF 96365, which prevents the capacitative calcium entry that occurs after intracellular store depletion in endothelial cells (Dolor et al., 1992). SKF 96365 (100 nM) significantly (P < .05) decreased the permeability of the BBB to [14C]sucrose after 6 h of hypoxia/aglycemia. These results further implicate calcium changes in the observed change in BBB function during hypoxia/aglycemia.

Because [14C]sucrose passage represents only the paracellular route of CNS entry across the BBB, experiments were performed to investigate whether receptor-mediated endocytosis is effected by hypoxia. Hypoxia has been shown to dramatically increase the non-specific transport of blood-borne proteins to the brain (Plateel et al., 1997), and this group showed a 10-fold increase in the permeability of albumin across cultured endothelial cells after 48 h of hypoxia. To measure receptor-mediated endocytosis during normoxia and hypoxia, we used DPDPE, a δ-opioid receptor agonist that accumulates in the brain via a saturable mechanism that is most likely receptor-mediated endocytosis (Williams et al., 1996; Egleton and Davis, 1999). Insulin also was chosen because BBMECs have been shown to express apical receptors that are involved in the processing and transport of blood-borne insulin across the BBB (Miller et al., 1994). [3H]DPDPE transport was found to be significantly reduced (P < .05) after 48 h of hypoxia. [125I]Insulin transport across the BBMECs after 48 h of hypoxia was found to be reduced, although this decrease was not statistically significant compared with normoxic BBMECs. This small reduction in receptor-mediated endocytosis may be due to ATP depletion in hypoxic BBMECs because endocytotic transport is an energy-dependent process. HPLC analysis of samples from the receiver chamber after 120 min of BBMVEC exposure showed that both peptides remained intact in our system (>90%).

Interestingly, the uptake of [14C]sucrose and [125I]insulin into BBMECs was found to be unaffected by 6 h of hypoxia/aglycemia, yet the uptake of [3H]DPDPE was found to be 1.5 times higher after 6 h of hypoxia/aglycemic exposure compared with normoxia. These data are interesting for DPDPE because the presumed transport (endocytosis and exocytosis) of intact compound is down-regulated with hypoxia, yet hypoxia/aglycemic exposure increases the endocytosis of this stable peptide agonist into endothelial cells.

Cytosfluorescence experiments were performed to examine the role of cytoskeletal elements in cerebral endothelial permeability during hypoxia. When cells were grown in normoxic conditions, FITC-phalloidin staining of endothelial cell F-actin displayed a marginal band distribution as well as a spindle-shaped morphology. After 24 h of hypoxia, endothelial cells showed no significant change in morphology or distribution of F-actin. Forty-eight hours of hypoxia resulted in a more diffuse, cytosolic distribution of F-actin, as well as a cuboidal cell shape. Similar experiments were performed under conditions of hypoxia/aglycemia. BBMECs maintained their characteristic morphology at up to 3 h of hypoxia/aglycemia, and at 6 h of hypoxia/aglycemia, we observed a slight change in F-actin distribution, similar to that observed at 48 h of hypoxia. These cytosfluorescence experiments demonstrate that the integrity of endothelial actin filaments is important for maintenance of BBB integrity because changes in the distribution of F-actin and BBB permeability to [14C]sucrose occur at the same time after hypoxia.

In conclusion, the results of the present work have shown that endothelial cells are sensitive to short durations of hypoxia/aglycemia. Hypoxia/aglycemia exposures resulted in a shorter time required (3–6 h) for a corresponding increase in the permeability of [14C]sucrose across BBMECs. Changes in endothelial cell calcium flux may be the biochemical mechanism responsible for the permeability change observed after hypoxia/aglycemia. Immunocytochemical experiments also revealed a change in the distribution of F-actin from a marginal band distribution during normoxia to a more diffuse cytosolic distribution with hypoxia and hypoxia/aglycemia. These experiments suggest that changes in BBB permeability may be mediated by changes in F-actin structure caused by calcium flux. This may have a direct effect on the presence of tight junctions in BBMECs because F-actin has been localized to the cytoskeletal domain of tight junctions (Lutz and Siahaan, 1997b). Calcium also may be involved in modulating tight junctional permeability via E-cadherin, which is a calcium-dependent adhesion molecule recently identified as the predominant adhesion molecule in cultured BBMECs (Lutz and Siahaan, 1997a; Pal et al., 1997). Future work will investigate the effect of hypoxia/aglycemia on the function and expression of E-cadherin.

This work provides evidence that the BBB is sensitive to hypoxia/aglycemia at short exposures and that changes in endothelial cell calcium flux may be responsible for these structural and functional variations in the BBB during ischemic stroke. Data from these and future studies will allow us to better define some of the non-neuronal mechanisms that contribute to the pathology of stroke.

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References


Send reprint requests to: Thomas P. Davis, Ph.D., Department of Pharmacology, University of Arizona College of Medicine, 1501 N. Campbell Ave., Tucson, AZ 85724. E-mail: davistp@u.arizona.edu