A functional role for sodium dependent glucose transport across the blood-brain barrier during oxygen glucose deprivation

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ABSTRACT

In the current study we determined the functional significance of sodium dependent/independent glucose transporters at the neurovasculature during oxygen glucose deprivation (OGD). Confluent brain endothelial cells cocultured with astrocytes were exposed to varying degrees of in vitro stroke conditions. Glucose transporter 1 (GLUT1) and sodium glucose cotransporter (SGLT) activity were investigated by luminal membrane uptake and transport studies using [3H] D-glucose and also by [14C] alpha methyl D-glucopyranoside (AMG), a specific, nonmetabolized substrate of SGLT. In vivo middle cerebral artery occlusion (MCAO) experiments were tested to determine if blood-brain barrier (BBB) SGLT activity was induced during ischemia. Increases in luminal D-glucose and AMG uptake and transport were observed with in vitro stroke conditions. Specific inhibitor experiments suggest a combined role for both SGLT and GLUT1 at the BBB during OGD. A time dependent increase in the uptake of AMG was also seen in mice exposed to permanent focal ischemia and this increase was sensitive to SGLT inhibitor, phlorizin. Infarct and edema ratio during ischemia were significantly decreased by the inhibition of this transporter. These results show that both GLUT1 and SGLT play a role at the BBB in the blood-to-brain transport of glucose during ischemic conditions and inhibition of SGLT during stroke has the potential to improve stroke outcome. Pharmacological modulation of this novel BBB transporter could prove to be a brain vascular target in stroke.
INTRODUCTION

The central nervous system is protected by three main physiological cell barriers which consist of arachnoid epithelium, the choroid plexus epithelium and the brain endothelium which form blood central nervous system interface. The brain itself is protected by brain endothelial cells which restrict the passage of many substances into and out of the brain, forming a selective blood brain barrier (BBB). For example various transporters are expressed at the BBB on both the luminal (blood facing) and the abluminal (brain facing) surfaces of the neurovascular barrier (Kumagai et al., 1995) such as glucose transporter, Na,K-ATPase, Na,K,2Cl-cotransporter, iron bound transferrin receptor mediated transporter. These all play a vital role in the transport of nutrients and ions and endogenous substances into and out of the brain. Transporter expression changes during disease states and overexpression or underexpression of some transporters occur either on the luminal or abluminal sides. For example increased luminal glucose transporter (GLUT1) density occurs with hypoglycemia (Simpson et al., 1999), increased density of Na,K,2Cl-cotransporter on the luminal (O'Donnell et al., 2004) and abluminal side (Abbruscato et al., 2004) occurs with stroke conditions, decreased activity of Na,K-ATPase on the abluminal side occurs with oxygen glucose deprivation (OGD) (Kawai et al., 1996; Abbruscato et al., 2004). It is apparent that the neurovascular unit does not simply function as a static barrier, yet has the ability to adapt during pathological states such as ischemia by its ability to transport ions and nutrients into and out of the brain.

Glucose is a major energy substrate for mammalian brain metabolism and a continuous supply of glucose is required for neuronal function. Under conditions of hypoxia, optimum glucose levels are needed to maintain low reactive oxygen species levels and high cell viability in primary cultured neurons (Shi and Liu, 2006). The major transporter through which
glucose gains access through BBB is the 55 kDa form of the facilitative glucose transporter protein GLUT1, which is independent of insulin (Harik et al., 1994). GLUT1 is known to be modulated by many pathophysiological conditions, such as Alzheimer’s disease where decreased density of GLUT1 is observed (Kalaria and Harik, 1989). An increase in brain glucose transporter capillary density was observed in chronic hypoxia (Harik et al., 1995), hypoglycemia (Kumagai et al., 1995) and ischemia (Harik et al., 1994). It is apparent that the BBB can increase or decrease nutrient transport depending on the pathophysiological state of the central nervous system.

Sodium glucose cotransporter (SGLT) is another glucose transporter which contributes to nutrient transport. SGLT was originally characterized in kidney proximal tubule epithelial cells and is known to be expressed more on the apical surface of the kidney and on the brush border membrane of the intestine (Wright, 2001). SGLT1, which transports 2Na⁺/glucose (Mackenzie et al., 1998) is expressed in the intestine epithelial cells and both SGLT1 and SGLT2 (which transports 1Na⁺/glucose (Mackenzie et al., 1996)) are expressed in kidney epithelial cells. Additionally SGLT1, the 70 to 75 kDa high affinity isoform, was shown to be expressed in neurons and to be upregulated during conditions such as metabolic stress when there is a decrease in D-glucose content (Poppe et al., 1997). SGLT, like GLUT1, has also been shown to be present on the brain artery endothelial cells and its importance was suggested to be in the maintenance of glucose levels in the arteries during the conditions of stress such as hypoglycemia (Nishizaki and Matsuoka, 1998). Immunodetection of SGLT1 was demonstrated at the BBB, and was shown to be upregulated after brain ischemia and reperfusion (Elfeber et al., 2004a). Recently the mRNA encoding for SGLT2 was also shown to be present and enriched in
isolated rat brain microvessels (Enerson and Drewes, 2006). However a functional role for SGLT has not been established in the brain.

In the present study using an *in vitro* model of varying degrees of OGD to mimic brain ischemia at the BBB (Abbruscato and Davis, 1999) and an *in vivo* model of middle cerebral artery occlusion, we demonstrated a functional role for SGLT in the blood to brain movement of glucose.
METHODS

Materials

$[^3]H$ D-Glucose (specific activity of 21.4 Ci/mmol) $[^3]H$ Mannitol (specific activity of 15-30 Ci/mmol) and $[^14]C$ alpha methyl D-glucopyranoside (specific activity of 250-350 mCi/mmol), was purchased from Perkin Elmer life Sciences (Boston, MA). $[^14]C$ Sucrose (specific activity of 625 mCi/mmol) was purchased from American Radiolabelled Chemicals, Inc (St. Louis, MO) Transwell™ Cell Culture assembly with polyester membrane inserts (0.4 µm pore size, 12mm diameter) were obtained from Corning Costar Corporation (Cambridge, MA). SGLT inhibitor phlorizin was purchased from Sigma Aldrich (St. Louis, MO) and GLUT1 inhibitor phloretin was purchased from Calbiochem (La Jolla, CA)

Cell Culture

Primary bovine brain microvascular endothelial cells (BBMECs) were isolated from fresh bovine brains as previously described (Abbruscato and Davis, 1999). In vitro BBMECs have been used extensively to model the BBB and mimic in vivo BBB transport and enzymatic activity. These cells are a pure culture of endothelial cells, free of astrocytes, neurons and pericytes and exhibit high transendothelial electrical resistance and polarity of expression of transporters (Abbruscato and Davis, 1999; Abbruscato et al., 2002; Abbruscato et al., 2004). First passage cells were seeded at a cell density of 50,000 cells/cm² on 12 well transwell plate inserts (0.4 µm pore size) coated with collagen and fibronectin. Factors released by astrocytes have been shown to mimic the in vivo conditions (Brillault et al., 2002), therefore on day 10 of BBMEC culturing C6 astroglioma cells (American Type Cell Collection, Rockville, MD) were seeded on the bottom well of BBMEC transwell™ inserts at a cell density of 40,000 cells/cm²
and were cultured to confluence for 48 hours. On day 12, BBMECs were ready for later experiments. All experiments described in these studies were reproduced with at least three separate isolates.

**Hypoxia (H), Hypoxia/Hypoglycemia (H/Hg), Hypoxia/Aglycemia (H/A)**

Confluent monolayer of BBMECs cocultured with astrocytes in the bottom well was exposed to varying degrees of oxygen and glucose deprivation. The concentration of glucose for normoxic conditions was 5.5mM. Hypoglycemia was induced by decreasing the glucose levels to 50% in the media and aglycemic conditions were induced by adding RPMI 1640 medium. Hypoxia was induced as previously described (Abbruscato and Davis, 1999; Abbruscato et al., 2004) by placing the cells in a custom made hypoxic polymer glove box (Coy Laboratories, Grass Lake, MI) which was infused with 95% N₂ and 5% CO₂ and the temperature was maintained at 37°C. The concentration of oxygen in the atmosphere was maintained at 0% and the PO₂ in the media was below 25 mmHg. Phlorizin, a phenolic glucoside, a competitive inhibitor of SGLT (Eskandari et al., 2005) and phloretin, a competitive inhibitor of GLUT1 (Betz et al., 1975) were used. A concentration of 50µmol/L (Nishizaki et al., 1995; Nishizaki and Matsuoka, 1998) was used for both the inhibitors. Bumetanide at a concentration of 20µmol/L was used to inhibit Na,K,2Cl-cotransporter (Abbruscato et al., 2004).

**Glucose Uptake Studies**

After H, H/Hg, HA exposure, [³H] D-glucose or [¹⁴C] AMG were added to the luminal side and the plates were rotated for 15 minutes and were then washed thrice with ice cold 0.1 M Tris buffer. BBMECs were solubilized with 1% Triton X-100 and the radioactivity present was
determined by counting the samples in a Beckman LS 6500 liquid scintillation counter (Beckman Instruments, Inc, Fullerton, CA). Protein content was calculated by using the detergent compatible BCA assay (Pierce Chemical, Rockford, IL) (Abbruscato et al., 2004). For studies using inhibitors, phlorizin or phloretin or a combination of both the inhibitors was added to the luminal side. Phloretin and phlorizin sensitive uptake were considered GLUT1 and SGLT activity respectively. For sodium dependent uptake studies, sodium was replaced by mannitol to maintain consistent osmolarity.

**Western Blotting Analysis**

The protein was isolated from BBMECs using TRIREAGENT<sup>LS</sup> at 0.4 ml/100 cm<sup>2</sup> of culture surface. Protein concentration was determined by using the pierce BCA assay kit and total cell membrane was denatured in Laemmli buffer, with β-mercaptoethanol, at 95°C for 5 min, resolved in SDS-PAGE (20 ug protein/lane), transferred to PVDF membrane (Amersham Biosciences Inc., NJ), and the PVDF membrane was incubated with the primary rabbit polyclonal human SGLT1 antibody (hSGLT1) 1:1000 over night at 4°C or a primary rabbit polyclonal β-actin antibody (1:4000) for 2 hours, followed by secondary antibody (1:5000) (goat anti-rabbit IgG-horse radish peroxidase) 2 hours at room temperature and the protein band was detected by enhanced chemiluminescence detecting reagents, Western Lightening (Amersham Biosciences Inc., Piscataway, NJ) (Sabolic et al., 2006).

**Transport Studies**

Transport studies were performed to determine the role of GLUT1 and SGLT on the blood to brain transport of glucose. Confluent BBMECs with astrocytes in the bottom well were
exposed to H/A conditions for 12 hours. [3H] D-glucose, [14C] AMG and [14C] sucrose transport studies were done as previously described (Weber et al., 1993; Abbruscato et al., 1996; Abbruscato and Davis, 1999). For studies using inhibitors, phlorizin or phloretin were added to both the luminal and the abluminal side to inhibit the transporters on both sides. For studies using Na, K, 2Cl-cotransporter inhibitor, bumetanide was added to the abluminal side to inhibit the transporter on abluminal side. The results were expressed as a permeability coefficient with the sucrose (the internal control for paracellular leakiness) space subtracted from the [3H] D-glucose or [14C] AMG values. Apical to basolateral permeation of [14C] sucrose and [3H] glucose were calculated by $P_{app} = \frac{\frac{dQ}{dt} \times 1}{A \cdot C_0}$ (Dehouck et al., 1992), where $\frac{dQ}{dt}$ is the flux of radiolabelled compounds (dpm/sec), A is the surface area available for transport, $C_0$ is the original donor concentration (dpm/ml) of the radioactive compounds.

**Middle Cerebral Artery Occlusion**

All studies were approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Permanent middle cerebral artery occlusion (MCAO) was performed on CD-1 mice (20-22g) which were anesthetized with 4% isoflurane by inhalation and maintained at 1% isoflurane in a nitrous oxide/oxygen 70/30 mixture. An incision was made in the skin and the left occipital and superior thyroid arteries, branches of external carotid artery and the pterygopalatine, a branch of the internal carotid artery were cauterized. A suture of 21 mm 5-0 nylon monofilament was introduced through the left external carotid artery, internal carotid artery, and finally to the middle cerebral artery (Mdzinashvili et al., 2005). A successful occlusion was verified by the
sudden drop of the blood flow by 10-15% of the basal flow as monitored by the laser Doppler flowmetry. After successful occlusion, the monofilament was secured in place with ligature and the skin incision was closed by surgical clips. The permanent MCAO was maintained for a period of 1, 3, 6 or 12 hours. Following MCAO, mice were anesthetized and 2 µci of $[^{14}\text{C}]$ AMG or $[^{14}\text{C}]$ sucrose (the internal control for paracellular leakiness) was injected through the femoral artery. After 30 minutes, 0.9% saline was infused through the heart for 1 min before decapitation, to wash the brain’s vascular space free of blood so that any radiolabelled substrate trapped in the vasculature would be removed. The perfusion was considered successful when the brain blanched completely and then tissues were processed as previously described (Weber et al., 1991). For studies using the inhibitor, the mice were pretreated with an intraperitoneal dose of 200mg/kg body mass phlorizin (dissolved in 1,3-propanediol) 1 hour before injecting AMG (Bormans et al., 2003; Elfeber et al., 2004b).

**AMG influx rate constant**

For the measurement of AMG uptake during 6 hours of permanent focal ischemia, mice received an IV bolus injection of $[^{14}\text{C}]$ AMG and blood was collected at different time intervals. $[^{3}\text{H}]$ mannitol, a plasma volume marker was injected 2 minutes before the sacrifice. A terminal blood sample was collected at the end of 30 minutes, the mice were deeply anaesthetized and decapitated. The brain was removed immediately, blotted dry and weighed. Both the blood and tissue samples were placed in a scintillation vial and the samples were solubilised with 1ml of tissue solubilizer and were then counted on a dual labelled scintillation counter. The influx rate constant $K_{in}$ (Kawai et al., 1998) was calculated as

$$K_{in} = \frac{C_{br}(T)}{AUC_{0-T}}$$  \hspace{1cm} (1)
\[ C_{br} (T) = C_{tot} (T) - [PV* C_{pl} (T)] \quad (2) \]

\( C_{br} (T) \) - Brain tissue concentration accumulated at time T, AUC \( 0-T \) (Area under the curve) - Integral of the plasma concentrations from time = 0 to time = T, PV= Plasma volume, \( C_{pl} (T) \) - final tracer plasma concentration. For studies using the inhibitor, the mice were pretreated with an intraperitoneal dose of 200mg/kg body mass phlorizin (dissolved in 1,3-propanediol) 1 hour before injecting AMG (Bormans et al., 2003; Elfeber et al., 2004b).

**Determination of Infarct and Edema Ratio**

After 6 hours of MCA occlusion, mice were deeply anesthetized, decapitated and the brain was cut into 1-mm thick coronal block slices. The brain slices were immersed in 1% solution of 2, 3, 5-triphenyltetrazolium chloride (TTC) in normal saline at 37°C for 20 min, and then fixed in 4% paraformaldehyde at 4°C. TTC stains viable brain tissue dark red based on intact mitochondrial function whereas infarcted tissue areas remain unstained (white). The size of infarct was calculated using an image analysis system (Image J 1.30 and Scion Image version Beta 4.0.2, NIH, Rockville, MD, USA). and infarct and edema ratios of hemispheric areas were calculated as previously described (Mdzinarishvili et al., 2005). Brain infarct was calculated as the ratio of infarct to the total brain ratio and brain edema (brain swelling) was quantified by comparing the area of the ipsilateral (ischemic) hemisphere to the contralateral (non ischemic) hemisphere. For studies with SGLT inhibitor phlorizin (200 mg/kg) (or vehicle 1,3 propane diol) was given intraperitoneally 1 hour after the induction of focal ischemia and the animals were sacrificed at 6\(^{th}\) hour.
Statistical Methods

All the data were expressed as mean ± SD and values were compared by student’s t-test or one way analysis of variance. This test was followed by Newman-Keuls multirange post hoc comparison of the means. Difference in $p$-values less than 0.05 were considered statistically significant.
RESULTS

Time Course Selection

In order to determine BBMEC sensitivity to H and H/A condition we performed studies at four different time points (1hr, 3hr, 6hr, and 12hr) and measured D-glucose uptake. 6 hours of H/A and 12 hours of H and H/A showed significant differences in the glucose uptake compared to the control (P<0.05, Figure 1). We observed a maximum uptake of the glucose at 12 hours of H/A. Glucose uptake peaks at 12 hours, therefore a time point of 12 hours was chosen in the later glucose in vitro uptake and transport studies.

Glucose and AMG Uptake Studies

Luminal [³H] D-glucose uptake studies were performed under control, H, H/Hg, and H/A conditions in the presence or absence of either GLUT1 inhibitor phloretin (50µmol/L) or SGLT inhibitor phlorizin (50µmol/L). The effect of the inhibitors was dose dependent at the BBB (data not shown). Under control conditions, with the inhibition of GLUT1, there was a significant decrease in the uptake of glucose, but with SGLT inhibition there was no change. With the inhibition of SGLT and GLUT1 independently during H, H/Hg, H/A conditions there was a significant decrease in the uptake (P<0.05, Figure 2A and 2B). The combination of both phlorizin and phloretin during H/A significantly reduced glucose uptake compared to each inhibitor by itself (P<0.05, Figure 2A and 2B). Control values averaged 1.4 ± 0.178 X 10⁻⁵ nmol/mg/min for D-glucose uptake. [³H] D-glucose uptake was also confirmed to be sodium dependent under H/A conditions and not sodium dependent under control conditions (P<0.05, Figure 2C). Furthermore luminal [¹⁴C] AMG (nonmetabolizable substrate) uptake studies were carried out in the presence or absence of phlorizin. We observed that under control conditions,
there was no significant change in the $^{14}$C AMG uptake in the presence of phlorizin. However, in H, H/Hg and H/A conditions, phlorizin caused a significant decrease in AMG uptake (P<0.05, Figure 3B). The viable cell count of BBMECs after OGD, in the presence or absence of inhibitors, remained constant throughout the experiment as confirmed by trypan blue exclusion (data not shown).

Immunoreactivity for SGLT1

Western blot analysis on the BBMECs total cell lysate confirmed immunoreactivity at 75 kDa in control, H, H/Hg, H/A conditions, which is consistent for SGLT1 (Sabolic et al., 2006) (Fig 3A). In all experiments we observed increased immunoreactivity for SGLT1 in conditions of oxygen and glucose deprivation.

Glucose and AMG Transport Studies

Luminal to abluminal transport studies were performed across confluent BBMEC monolayer (Fig 4). Control values for $[^3]$H D-glucose permeability averaged $280 \times 10^{-6} \pm 79.7 \times 10^{-6}$ mm/sec, which compares well with literature values of $235 \times 10^{-6}$ mm/sec for apparent permeability done in an rat in vivo model (Gjedde, 1981). Control values for $[^3]$H D-glucose was significantly greater than $^{14}$C sucrose values which averaged to $346 \times 10^{-7} \pm 48.8 \times 10^{-7}$ mm/sec. $[^3]$H D-glucose transport in control conditions was sensitive to phloretin but not phlorizin suggesting the absence of SGLT mediated blood to brain glucose transport during control conditions. There was a significant increase in total glucose transport with H/A treatment and this increase in glucose transport was sensitive to the presence of phlorizin or phloretin suggesting role of both GLUT1 and SGLT in blood to brain transport of glucose.
The magnitude of inhibition by phloretin was not different between control and H/A. Furthermore, $^{[14]}$C AMG transport studies were carried out in the presence or absence of phlorizin. Control values of $^{[14]}$C AMG averaged to $262 \times 10^{-7} \pm 81.8 \times 10^{-7}$ mm/sec, which was not significantly different from $^{[14]}$C sucrose values. There was a significant increase in $^{[14]}$C AMG transport with H/A and this was inhibited with phlorizin ($P<0.05$, Figure 4B).

$[^{3}\text{H}]$ D-glucose apical to basolateral transport was sensitive to the Na,K,2Cl-cotransporter inhibitor bumetanide in H/A conditions suggesting the reliance of abluminal SGLT on the Na,K,2Cl-cotransporter during OGD conditions ($P<0.05$, Figure 5).

**Blood to brain transport and influx rate constant of AMG**

Blood to brain transport of $^{[14]}$C AMG was measured in mice exposed to permanent focal ischemia for different time points of 1, 3, 6 and 12 hours. Control value averaged to $0.14 \pm 0.03$ % total $^{[14]}$C AMG injected/gram tissue. $^{[14]}$C AMG uptake values in sham operated animals were found to be no different from control animals. A significant increase in the uptake of $^{[14]}$C AMG was observed with all the time points tested for the total brain, 6 and 12 hours for the ipsilateral hemisphere and 12 hours for the contralateral hemisphere when compared to the control conditions ($P<0.05$, Figure 6A). Blood to brain transport of $^{[14]}$C sucrose, an impermeable marker was also measured in mice exposed to permanent focal ischemia for 12 hours. Control values averaged $0.132 \pm 0.016$ and 12 hours stroke value averaged to $0.122 \pm 0.04$ % total sucrose injected/gram tissue.

Further studies to measure the influx rate constant ($K_{in}$) of AMG were carried out at a time point of 6 hours in the presence of a vascular marker, mannitol (FW= 182.17) similar in size to AMG (FW= 194). A significant increase in the value of $K_{in}$ of $^{[14]}$C AMG (corrected for
vascular space with \(^3\)H mannitol) was observed at six hours of focal ischemia and this increase was significantly decreased with phlorizin (P<0.05, Figure 6B). The vehicle had no effect on AMG distribution.

**Brain Infarct and Edema Ratio**

The infarct and edema ratios measured in mouse brain subjected to focal ischemia were significantly reduced in animals treated with phlorizin in comparison to vehicle controls (P<0.05, Fig 7). The vehicle alone had no effect on the infarct and edema ratio.
DISCUSSION

Glucose is the primary energy source for the brain and in stroke conditions, where there is a need for more glucose due to rapid oxygen depletion; regulation of brain glucose transport becomes an important factor. Glucose is known to be transported at the BBB by GLUT1, a saturable transporter which is independent of energy or ion substrates, but an additional sodium dependent glucose transporter was also suggested to play a role in blood to brain movement of glucose during pathophysiological conditions.

The significance and the novelty of this study is the functional characterization of SGLT at the BBB, which is previously reported to be present mostly in kidney and the intestines (Wright, 1993; Wright, 2001). Although studies have demonstrated SGLT1 immunoreactivity at the BBB (Elfeber et al., 2004a) and its upregulation with ischemia, our studies are the first to show a functional role for SGLT at the BBB, with varying degrees of in vitro and in vivo ischemic conditions. In our studies we used confluent BBMECs cocultured with C6 astrocytes, to best mimic the association of endothelial cells with the astrocytes at the BBB (Brillault et al., 2002). We investigated glucose uptake into the BBMECs with H, H/Hg, H/A conditions, which was used to model varying degrees of in vitro stroke conditions (Abbruscato and Davis, 1999). Luminal uptake experiments suggest the presence of GLUT1 and absence of SGLT activity in control conditions but suggest a role for both these transporters during H, H/Hg, H/A conditions. This was further confirmed by luminal AMG (nonmetabolisable substrate) uptake studies and positive immunoreactivity of SGLT1 (Figure 3). Additionally, we determined that the combination of phlorizin and phloretin during H/A significantly reduced glucose uptake compared to each inhibitor by itself (Figure 2A and 2B), thus showing the specificity of each inhibitor for their respective transporter. Further studies investigated the effects of H/A on D-
glucose and [14C] AMG transport and our results strongly suggest the involvement of SGLT in the blood to brain transport of glucose during OGD, an in vitro condition that mimics neurovascular ischemia (Abbruscato et al., 2004)(Figure 4).

Blood to brain transport across the neurovascular unit requires movement across both the luminal and abluminal surface of the brain endothelial cell. SGLT is also known to have the ability to function in reverse mode when the intracellular sodium concentration is increased and it can transport glucose from the interior of the cell into the extracellular fluid (Eskandari et al., 2005). The presence of a higher affinity sodium dependent transporter on the abluminal surface of the BBB was suggested in isolated plasma membranes from bovine microvascular endothelial cells (Lee et al., 1997), although these experiments were not confirmed using in vivo models. Na,K,2Cl-cotransporter which transports 1Na+, 1K+ and 2Cl- ions, pumps sodium from the brain extracellular fluid into the cell and is upregulated during OGD conditions at the BBB on both luminal and abluminal sides (Abbruscato et al., 2004; O'Donnell et al., 2004). This BBB Na, K, 2Cl-cotransporter may provide a sodium gradient to fuel sodium dependent brain glucose transport during H/A by SGLT from the inside of brain endothelial cells to the brain extracellular fluid. Therefore to study the possible dependence of SGLT on Na,K,2Cl-cotransporter for intracellular sodium, apical to basolateral glucose transport studies were tested. We observed an increase in glucose transport with 12 hours of OGD that was sensitive to Na,K,2Cl-cotransporter inhibitor bumetanide, suggesting the reliance of SGLT on Na,K,2Cl-cotransporter (Figure 5). We also confirmed the presence of SGLT activity at the BBB with different levels of in vitro ischemia. Hypoxia alone or combined with hypoglycemia or aglycemia models the various stages of nutrient deficit going from the core to the penumbral regions. We determined that SGLT plays a role in the uptake and transport of glucose from blood to brain along with GLUT1.
To further strengthen the presence of SGLT at the BBB we designed focal ischemia experiments in mice under 12 hours. Our studies with $[^{14}\text{C}]$ sucrose, an impermeable marker, and studies in the literature have shown that significant paracellular BBB breakdown does not occur under 12 hours of permanent MCAO (Hatashita and Hoff, 1990). Additionally, increased expression of matrix metalloproteinase’s do not occur until after 12 hours MCAO (Romanic et al., 1998). Therefore, our experiments were conducted at time point’s ≤ 12 hours, where the paracellular barrier is believed to remain intact. A significant increase in the brain uptake of $[^{14}\text{C}]$ AMG was seen with all the time points in total brain, with 6 and 12 hours in the ipsilateral hemisphere and with 12 hours in contralateral hemisphere when compared to the control conditions (Figure 6A). These results are consistent with another group that observed a bilateral upregulation of SGLT1 immunoreactivity after MCAO (Elfèber et al., 2004a). We speculate that the observed changes in both ischemic and non-ischemic hemispheres at the 12 hour time point could be due to release of mediators into the circulation. Other researchers have also reported bilateral up- and down-regulation of brain specific proteins (Focking et al., 2006) including brain endothelial cell IL-1β (Zhang et al., 1998) with focal ischemia. Future investigations will decipher these interesting pathophysiologic results. Additionally, we observed that the influx rate constant, corrected for vascular space for $[^{14}\text{C}]$ AMG after 6 hours of focal ischemia was significantly decreased in the presence of phlorizin further strengthening the function of SGLT at the BBB (Figure 6B). These in vivo experiments help to verify the function of SGLT mediated transport at the BBB during ischemia and validate that it takes part in both apical and basolateral transport across the BBB. Since $[^{14}\text{C}]$ AMG has specificity for SGLT and not GLUT1 and is a non metabolizable substrate (Poppe et al., 1997; Bormans et al., 2003), this data strongly
supports that SGLT mediates a significant amount of glucose transport across the BBB during ischemia.

OGD induced SGLT activity could be detrimental during ischemia as it could cause more sodium and calcium influx and contribute to ischemic neuronal damage (Elfeber et al., 2004a). To test this possible hypothesis, we administered phlorizin 1 hour after focal ischemia and we observed a significant decrease in ischemia and edema ratios as judged by TTC staining of viable cells and from measurements of hemispheric enlargement after 6 hours of focal ischemia (Figure 7). We believe that this action of phlorizin on BBB SGLT may have several potential implications. For example, inhibition of SGLT could decrease the continued delivery of glucose which has been shown to be detrimental during ischemia due to cerebral acidosis and free radical production resulting from anaerobic metabolism of glucose. Another result of SGLT inhibition could be reduction of brain edema associated with ischemia since this transporter is known to transfer approximately 210 water molecules for each Na⁺ and glucose molecule (Wolkoff et al., 1998), therefore this could reduce edematous conditions associated with ischemia. Future investigations will decipher the neuroprotective mechanism(s) involved in SGLT inhibition at the neurovascular unit, as this transporter is known to be present on both the astrocytes and neurons (Poppe et al., 1997; Vega et al., 2006).

Our results suggest the presence of SGLT at the BBB. Based on the current data we proposed a working hypothesis for SGLT at the BBB (Figure 8). Na,K,2Cl-cotransporter (green) may provide a sodium gradient (blue arrows) to fuel sodium dependent brain glucose transport during ischemia by SGLT (yellow) from the inside of brain endothelial cells to the brain extracellular fluid. Additionally, the abluminal Na,K-ATPase (blue), which aids in the efflux of sodium from the brain endothelial cell into the brain extracellular fluid has been shown to be
deactivated during models of ischemia (Kawai et al., 1996; Abbruscato et al., 2004). The net efflux of sodium from brain endothelial cells is comparatively less, and this would further contribute to the accumulation of sodium in brain endothelial cells during stroke conditions. As a proposed operative mechanism in intestinal and kidney epithelial cells (Eskandari et al., 2005), SGLT in the brain might also have the capability to cotransport sodium and glucose into and out of the cells when provided with a sodium driving force, during conditions of OGD. Future immunohistochemical or electron microscopy experiments are needed to validate the expression polarity of SGLT at the BBB during ischemia.

In summary, we have determined the luminal activity of SGLT and confirmed its role in the transport of glucose from blood to brain with in vitro and in vivo conditions. Further we have confirmed its protective role in permanent focal ischemia. Future studies on the expression, regulation and inhibition of SGLT in vivo with temporary focal ischemia when BBB damage occurs more acutely would lead to further understanding as to how SGLT can be utilized as a therapeutic target in stroke and other CNS disorders. Future work is needed to determine the effect of modulating this transporter in association with tPA therapy. Modulating this novel neurovascular target possibly could provide a means to extend the therapeutic window of t-PA therapy by reducing the edematous condition and preventing the negative effects of hyperglycemia.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Time dependent studies of luminal glucose uptake into BBMECs cocultured with astrocytes. (A) hypoxia (B) hypoxia with aglycemia. Data represent mean ± standard deviation, n=5. *p<0.05 compared with the control using one-way analysis of variation and Newman-Keuls multiple comparison. H- hypoxia; H/A- hypoxia/aglycemia.

Figure 2. Effect of H, H/Hg, H/A on luminal glucose uptake into BBMECs with [³H] D-glucose in the (A) presence or absence of phloretin (B) presence or absence of phlorizin (C) Na⁺ dependence of BBMEC on [³H] D-glucose uptake with H/A. Data represent mean ± standard deviation, n=5. *p<0.05 compared with the control using one-way analysis of variation and Newman-Keuls multiple comparison. H- hypoxia, H/Hg- hypoxia/hypoglycemia, H/A- hypoxia/aglycemia, PHT- Phloretin, PHZ- Phlorizin.

Figure 3. (A) SGLT1 Immunoreactivity with H, H/Hg, H/A conditions (B) Effect of H, H/Hg, H/A on luminal [¹⁴C] AMG uptake into BBMECs in the presence or absence of phlorizin. Data represent mean ± standard deviation, n=5. *p<0.05 compared with the control using one-way analysis of variation and Newman-Keuls multiple comparison. H- hypoxia, H/Hg- hypoxia/hypoglycemia, H/A- hypoxia/aglycemia, PHZ- Phlorizin.

Figure 4. (A) Transport of [³H] D-glucose across BBMECs monolayer during H/A in the presence or absence of SGLT inhibitor phlorizin or GLUT1 inhibitor phloretin. (B) Transport of [¹⁴C] AMG across BBMEC monolayer during H/A in the presence or absence of SGLT inhibitor, phlorizin. Data represent mean ± standard deviation, n=5-6. *p<0.05 compared with the control.
using one-way analysis of variation and Newman-Keuls multiple comparison. H/A- hypoxia/aglycemia, PHT- Phloretin, PHZ- Phlorizin.

**Figure 5.** Modulation of BBB SGLT transport by bumetanide during H/A. Data represent mean ± standard deviation, n=4-5. *p<0.05 compared with the control using one-way analysis of variation and Newman-Keuls multiple comparison. H/A- hypoxia/aglycemia.

**Figure 6. (A)** Transport of [14C] AMG into mouse brain exposed to permanent focal ischemia for 1, 3, 6 or 12 hours. Control values averaged to 0.14 ± 0.03 % total [14C] AMG injected/gram tissue. (B) Influx rate constant (K_in) of [14C] AMG into mouse brain exposed to permanent focal ischemia for 6 hours. Data represent mean ± standard deviation, n=4-5. *p<0.05 compared with the control using one-way analysis of variation and Newman-Keuls multiple comparison.

**Figure 7.** Effect of phlorizin on infarct ratio (infarct area/brain slice area) and edema ratio (ipsilateral/contralateral hemisphere slice area) following 6 hour MCAO. Visual inspection shows that there is a striking attenuation of the ischemic area in the slices treated with Phlorizin. Data represent mean ± SEM of 5 independent determinations containing 5-6 slices each. *p<0.05 compared with the control using t-test.

**Figure 8.** Working hypothesis for enhanced sodium dependent glucose transport across the blood-brain barrier during ischemia. SGLT is activated at the BBB during ischemia and aids in the transport of glucose along with GLUT1 from blood to brain. Na, K, 2Cl-cotransporter which is activated on the luminal and the abluminal sides in conditions of low oxygen and glucose
could provide a driving sodium force (blue arrows) which would fuel SGLT for sodium coupled glucose transport into the brain extra cellular fluid. Additionally, when the abluminal Na⁺,K⁺-ATPase is deactivated at the BBB during H/A, there would be a decrease in the net efflux of sodium from the brain endothelial cell thus a driving sodium force for the net movement of glucose from the endothelial cell interior to the brain extra cellular fluid.
FIGURE 3A

SGLT1 75 kDa

β-actin

Control  H  H/Hg  H/A

SGLT1 Immunoreactivity (Arbitrary Units)

control  H  H/Hg  H/A

*
% control of $[^3H]$ D-glucose permeability

![Graph showing % control of D-glucose permeability across different conditions and concentrations. The graph includes bars for control, control+PHT, control+PHZ, H/A, H/A+PHT, and H/A+PHZ. Bars are labeled with percentages and error bars are present. Statistical significance indicated by asterisks (*) for certain conditions and concentrations.](image)
FIGURE 6B

![Graph showing treatment effects on $K_m$ values](image)

- Control
- 6 hour ischemia
- 6 hour ischemia + phloretin

$K_m$ (mL/g/min)

* indicates significant difference
**NORMAL BLOOD FLOW**

**BLOOD**

1. $\text{Na}^+$
2. $\text{Cl}^-$
3. $\text{K}^+$
4. Glucose

**ISCHEMIA**

1. $\text{Na}^+$
2. $\text{Cl}^-$
3. $\text{K}^+$
4. Glucose

**BRAIN**

1. $\text{Na}^+$
2. $\text{Cl}^-$
3. $\text{K}^+$
4. Glucose

**Key**
- GLUT1
- SGLT
- Na,K,2Cl⁻ Cotransporter
- Na K ATPase

1, 2, 3, 4 – ACTIVATED
5 – DEACTIVATED